UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Caracterización de los efectos neuroprotectores del cannabidiol en el daño cerebral hipóxico-isquémico neonatal

Characterizationof the neuroprotective effects of cannabidiol in newborn hypoxic-ischemic brain damage

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

María Ceprián Costoso

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José Martínez Orgado
Mª Ruth Pazos Rodríguez
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CERTIFICAN:

Que la presente Tesis Doctoral titulada: "Caracterización de los efectos neuroprotectores del cannabidiol en el daño cerebral hipóxico-isquémico neonatal/ Characterization of the neuroprotective effects of cannabidiol in newborn hypoxic-ischemic brain damage" presentada por María Ceprián Costoso, Licenciada en Biotecnología, para optar al título de Doctor por la Universidad Complutense de Madrid, ha sido realizada bajo su dirección y reúne todos los requisitos necesarios para ser juzgada.

Y para que así conste, y a los efectos oportunos, firman el presente certificado:

Dr. José Martínez Orgado Dra. Ma Ruth Pazos Rodríguez Dr. Javier Fernández-Ruiz

En Madrid a 30 de Octubre de 2018.

Abbreviations

2-AG	2-arachidonoylglycerol
ABHD12	α/β-hydrolase domain contain 12
ABHD6	α/β-hydrolase domain contain 6
AEA	*N*-arachidonoylethanolamine; anandamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
BBB	Brain blood barrier
BDNF	Brain Derived Neurotrophic Factor
BrdU	nucleoside 5-bromo-2'-deoxyuridine
cAMP	Cyclic adenosine monophosphate
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
CBD	Cannabidiol
CBF	Cerebral blood flow
COX2	Cyclooxygenase 2
CP	Cerebral Palsy
CRT	Cylinder Rearing Test
DAGL	Diacylglycerol-lipase
ECS	Endocannabinoid System
FAAH	Fatty acid amide hydrolase
FABPs	Fatty acid binding proteins
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial Cell-derived Neurotrophic Factor
Glu/NAA	Glutamate/N-acetylaspartate
GPCR	G protein-coupled receptor
H^+-MRS	Magnetic Resonance Spectroscopy
HI	Hypoxia-Ischemia
IGF-1	Insulin-like growth factor 1
IL	Interleukine
iNOS	inducible NO synthase
IP_3	1,4,5- triphosphate

Lac/NAA	Lactate/N-acetylaspartate
MAGL	Monoacylglycerol lipase
MCAO	Temporary middle cerebral artery occlusion
MCP1	Monocyte chemoattractant protein-1
mI/Cr	Myoinositol/Creatine
MRI	Magnetic Resonance Imaging
NAAA	NAE-hydrolyzing acidamidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPE	N-arachidonoyl phosphatidylethanolamine
NAPE-PLD	NAPE-phospholipase D
NE	Neonatal Encephalopathy
NFKβ	Nuclear actor kappa-light-chain-enhancer of activated B cells
NHIE	Neonatal Hypoxia-Ischemia Encephalopathy
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOR	Novel Object Recognition
OGD	Oxygen and glucose deprivation (*in vitro* model)
OPC	Oligodendrocyte progenitor cell
PAIS	Perinatal Arterial Ischemic Stroke
PBS	Phosphate Buffer Saline
PKA	Protein kinase A
PND	Postnatal day
PPAR	Peroxisome proliferator-activated receptors
preOL	Preoligodendrocye
ROS	Reactive oxygen species
SR2	SR144528
SVZ	Subventricular zone
TdT	Terminal deoxynucleotidyl transferase
TH	Therapeutic hypothermia
THC	Δ^9- tetrahydrocannabinol
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis induced ligand

TRPA1	Transient-receptor-potential-related A1
TRPV1	Transient receptor potential type V 1
WHO	World Health Organization

Summary

Summary

Neonatal hypoxic-ischemic (HI) brain injury is a severe condition which main deleterious consequence is cerebral palsy. Two main neonatal hypoxic-ischemic pathologies are described: Neonatal Hypoxia-Ischemia Encephalopathy (NHIE), a global, diffuse brain damage, and Perinatal Arterial Ischemic Stroke (PAIS), a focal HI insult which usually affects the left middle cerebral artery territory.

The primary cause of damage in NHIE and PAIS is the decrease of glucose and oxygen due to the depletion of brain blood flow, which induced the so-called "deadly triad", a combination of excitotoxicity, oxidative stress and inflammation that eventually leads to cell death and HI-induced brain damage. Oligodendrocytes are particularly sensitive to HI damage, mainly in its preoligodendrocyte stage, and eventually HI-induced hypomyelination. Astrocytes and microglia contribute actively promoting the "deadly triad" but are also key in the reparative response triggered after HI.

Nowadays only hypothermia is approved for moderate to severe NHIE treatment, while PAIS treatment is focused in treating the main symptoms. Therefore, there is an urgent need of new molecules that can improve NHIE and PAIS outcome and cannabidiol (CBD) has risen as a really promising drug. CBD is a phytocannabinoid with no psychostimulant effect that modulates excitotoxicity, inflammation and oxidative stress. Its administration reduces brain damage in neonatal models of HI in both rodents and piglets and is able to prevent myelin loss in adult mice models of demyelinating diseases. Although its mechanism is not clear, some works have pointed out that the CB_2 receptor, a cannabinoid receptor, might be involved in CBD neuroprotective actions.

Therefore, it is hereby hypothetized that CBD administration post-injury will exert short- and long-term protective effects in models of either neonatal global or focal HI, i.e. NHIE or PAIS respectively. To study this hypothesis four goals were set:

1. To characterize medium- and long-term CBD neuroprotective effects in a focal model of hypoxic-ischemic brain injury in newborn rats.
2. To analyze HI-induced long-lasting hypomyelination in White and Grey Matter and its prevention by CBD.
3. To assess whether CBD hypomyelination prevention is related with brain cell proliferation activation and glial response modulation.
4. To study the involvement of CB_2 receptor activation in CBD protective effects in a newborn rat model of hypoxic-ischemic brain damage.

In the focal model of HI, the ~~i~~administration of CBD 5mg/Kg was able to prevent the motor impairment induced by MCAO one week and one month after the insult. Along with this result, CBD animals showed lower levels of excitotoxicity, oxidative stress and cell death one week after damage, that agreed with the preserved neuronal population observed at both ages in MCAO-CBD animals *versus* MCAO-VEH. Although no differences in brain volume loss were observed by magnetic resonance imaging (MRI), CBD significantly reduced the perilesional hyperintense area which one month after the insult correlated with CBD-induced decreased astrogliosis. CBD administration was not only able to reduce MCAO-induced astro- and microgliosis, but also promoted an anti-inflammatory glial state, preserving astroglial function.

A decrease of myelin content was observed in both global and focal models one month after the insult. Global HI led to myelination impairment and axonopathy in both White and Grey Matter, although only in cerebral cortex a decrease of mature oligodendrocytes was observed. Hypomyelination correlated with HI-induced long-lasting sequelae. Specifically, a positive correlation was observed between White Matter MBP and hemiparesis, and cerebral cortex hypomyelination and memory impairment. When oligodendrocyte was studied, early death of oligodendrocytes was observed in both areas, followed by the restoration of cellularity seven days after the insult. CBD administration increased $Olig2^+$ cell density and prevented the decrease of $SOX10^+$ over the fourth and the sixth day after the insult. Besides, a more active differentiation of $Olig2^+$ population into astroglial lineage was observed in vehicle animals but not in CBD group. In agreement, CBD led to the reduction of astro- and microgliosis and of cytokines release, excitotoxicity and oxidative stress.

To study if CB_2 receptors were involved in CBD neuroprotection, we compared the effects of CBD and those of HU-308, a specific CB_2 agonist, when administered after HI. CBD exerted a remarkable neuroprotective effect reducing motor impairment, brain volume damage and brain TNFα concentration increase. By contrast, HU-308 only partially reduced motor impairment, showing an additive effect when was co-administrated with CBD, but with no effect on the other tests. The co-administration of CBD and SR144528, a CB_2 antagonist, reversed some of CBD protective effects.

In conclusion, HI insult led to brain injury resulting in long-lasting motor and cognitive sequelae that correlated with acute cell death in a manner related with excitotoxicity, increased oxidative stress and inflammation. HI triggered a strong micro- and astrogliosis characterized by the increase in the number of cells and its transformation into an activated state as well as the loss of astrocytic function, and eventually leading to the development of a glial scar, corresponding

to an increased hyperintense area observed one month later. Therefore, HI led to a remarkable decrease on neuronal population and myelin content. Particularly, global HI was associated to White and Grey Matter axonopathy and hypomyelination, which correlated with motor and cognitive disabilities in a region-specific manner, probably because of the different oligodendrocyte maturative stage.

CBD administration post-HI was neuroprotective in the short- and long-term, reducing brain volume loss and motor and cognitive sequelae. CBD neuroprotection was linked to the preservation of neuronal population by reducing HI-induced cell death in a manner related with the reduction of excitotoxicity, oxidative stress and inflammation. CBD administration reduced HI-induced astro- and microgliosis, promoting its non-activated state and preserving astrocyte function as well as oligodendrocyte lineage promotion over astroglial's. Furthermore, CBD blunted HI-induced hypomyelination and axonopathy in a region-specific manner, preserving proper oligodendrocyte maturation and myelination. Finally, CB_2 receptor activation was somehow involved in CBD neuroprotection, likely by its indirect activation, although other ways might also participate in CBD effects.

Resumen

Resumen

El daño hipóxico-isquémico (HI) neonatal es una patología grave cuya principal secuela es la parálisis cerebral. Dos de las principales patologías HI neonatales son la Encefalopatía Hipóxico-Isquémica Neonatal (NHIE)[1], caracterizado por un daño cerebral global y difuso, y el Infarto Isquémico Arterial Perinatal (PAIS), un daño HI focal que afecta principalmente al territorio de la arteria cerebral media izquierda.

El origen del daño HI es el descenso de glucosa y oxígeno causado por la disminución del flujo cerebral, que induce la llamada "triada mortal" (excitotoxicidad, estrés oxidativo e inflamación), que conduce a la muerte celular y al daño cerebral. Al ser los oligodendrocitos, especialmente en su etapa de pre-oligodendrocitos, especialmente sensibles al daño la HI produce una hipomielinización. Los astrocitos y la microglia promueven activamente la "triada mortal " pero también son fundamentales en la respuesta reparativa generada por la HI.

La hipotermia es el único tratamiento actual para la NHIE moderada o severa, mientras que en el PAIS el tratamiento es sintomático. Por ello, es perentoria la búsqueda de nuevas moléculas, entre las que destaca el cannabidiol (CBD). Este fitocannabinoide no psicoactivo modula la excitotoxicidad, la inflamación y el estrés oxidativo. Su administración tras la HI neonatal reduce el daño cerebral en roedores y lechones, y previene la pérdida de mielina en modelos de desmielinización en roedores adultos. Aunque su mecanismo no está claro, algunos trabajos han demostrado que el receptor cannabinoide CB_2 puede mediar alguno de sus efectos protectores.

En consecuencia, la hipótesis es que la administración de CBD tras el daño HI neonatal será neuroprotectora a corto y largo plazo tanto en un modelo global como focal, NHIE o PAIS respectivamente. Para ello, se han establecido cuatro objetivos:

1. Caracterización a medio y largo plazo del papel neuroprotector del CBD en un modelo focal del daño HI en rata neonatal.
2. Análisis de la hipomielinización inducida por la HI en Sustancia Gris y Blanca, y su prevención por el CBD.
3. Estudiar si el efecto protector del CBD en la hipomielinización está relacionado con la activación de la proliferación de células cerebrales o la modulación de la respuesta glial.
4. Analizar el papel de la activación del receptor CB_2 en los efectos protectores del CBD en un modelo de daño HI global en rata neonata.

[1] Las abreviaturas hacen referencia a la denominación inglesa de la patología, como se pueden encontrar en el apartado de abreviaturas.

En el modelo de HI focal se observó que el CBD a 5 mg/Kg prevenía el daño motor asociado al MCAO una semana y un mes después de la lesión. Los animales tratados con CBD mostraron menores niveles de excitotoxicidad, estrés oxidativo y muerte celular una semana después del daño, preservando la población neuronal a ambas edades. Aunque el análisis del volumen de lesión por resonancia magnética nuclear no mostró diferencias, el CBD redujo el volumen del área hiperintesa perilesional, correlacionándose con una menor astrogliosis un mes después de la lesión. El CBD no solo redujo la micro- y la astrogliosis sino también promovió un fenotipo antinflamatorio, preservando la función astroglial.

En ambos modelos se observó un descenso de mielina un mes después de la lesión. La HI global produjo una axonopatía y una mielinización deficiente en Sustancia Blanca y Gris, aunque el número de oligodendrocitos maduros sólo disminuyó en la corteza cerebral. La hipomielinización se asoció con las secuelas a largo plazo de la HI, observándose una correlación entre la MBP de Sustancia Blanca y la hemiparesis, y la hipomielinización de corteza cerebral y la alteración de memoria. Se observó una muerte temprana de oligodendrocitos, seguida de un restablecimiento de su celularidad siete días después de la HI. La administración de CBD incrementó la densidad celular $Olig2^+$ y previno el descenso de la $SOX10^+$ durante el cuarto al sexto día después de la HI. Ésta indujo una diferenciación más activa hacía el linaje astroglial de las células $Olig2^+$, efecto prevenido por CBD. Asimismo, el CBD redujo la astro- y la microgliosis, la liberación de citoquinas, el estrés oxidativo y la excitotoxicidad inducidos por la HI.

Para estudiar si el receptor CB_2 estaba implicado en la neuroprotección del CBD, comparamos los efectos del CBD y del HU-308, agonista específico del receptor CB_2. El CBD demostró un notable efecto neuroprotector y antinflamatorio. Sin embargo, el HU-308 sólo redujo parcialmente el daño motor, mostrando un efecto aditivo con el CBD, pero sin efecto en el resto de pruebas. La coadministración de SR144528, antagonista de CB_2, previno algunos de los efectos protectores del CBD.

Como conclusión, la HI indujo daño cerebral con secuelas motoras y cognitivas a largo plazo que se correlacionó con la muerte celular aguda asociada a excitotoxicidad, aumento del estrés oxidativo e inflamación. La HI indujo micro- y astrogliosis caracterizada por el aumento del número de células y su transformación a estado activado y la pérdida de función astrocitaria, que finalmente indujo la cicatriz glial asociada con el aumento del área hiperintensa observado un mes después. En consecuencia, la HI produjo un marcado descenso de la población neuronal así como hipomielinización y axonopatía de la Sustancia Gris y Blanca, cada área específicamente

asociada con determinadas secuelas motora y cognitiva, probablemente por el diferente estado madurativo del oligodendrocito.

La administración del CBD fue neuroprotectora a corto y largo plazo, reduciendo la pérdida de volumen cerebral y las secuelas motoras y cognitivas, preservando la población neuronal al reducir la muerte inducida por la HI en relación al descenso de excitotoxicidad, estrés oxidativo e inflamación. La administración del CBD redujo la micro- y astrogliosis, promoviendo su estado no-activado, preservando la función astrocitaria y el predominio del linaje oligodendrocítico sobre el astrocitario. Además, el CBD previno la hipomielinización y axonopatía, preservando la maduración del oligodendrocito y la mielinización. Finalmente, la activación del receptor CB_2 participó en la neuroprotección del CBD, probablemente mediante su activación indirecta, aunque otros mecanismos podrían estar también involucrados.

Introduction

1.NEONATAL PERIOD

The neonatal period has been defined by the World Health Organization (WHO) as the period ranging from birth to the end of fourth week of life (28 completed days) (Ryninks et al., 2006) and is the most vulnerable in infants' life. Approximately 0.662 million neonates die because intrapartum complications every year in the world (Liu et al., 2015), being the WHO estimation that 8% of children deaths under 5 years-old are caused by asphyxia at birth (Bryce et al., 2005). Remarkably, although global childhood mortality showed a reducing trend from 1990 to 2013, neonatal and specially early neonatal (less than seven days after birth) mortality rates have barely decreased (Lehtonen et al., 2017). This is specially worrying in developing countries, where death is the main consequence of intrapartum asphyxia (Lee et al., 2013; Liu et al., 2015). Whereas the improvement of health care system in developed countries resulted in increased survival rate (Lee et al., 2013; Lehtonen et al., 2017), it has also increased the incidence of long-lasting sequelae, being the major one cerebral palsy (CP). CP is considered the most prevalent childhood motor disability (Johnston and Hoon, 2006), with an average lifetime cost of 921.000 $ per person (Centers for Disease Control and Prevention, 2004; Johnston and Hoon, 2006).

The clinical term that englobes these complex neonatal pathologies is Neonatal Encephalopathy (NE). NE is defined by the American Academy of Pediatrics as "a clinically defined syndrome of disturbed neurologic function in the earliest days of life in an infant born at or beyond 35 weeks of gestation, manifested by a subnormal level of consciousness or seizures, and often accompanied by difficulty with initiating and maintaining respiration and depression of tone and reflexes" (D'Alton et al., 2014). A major cause of NE is an hypoxia-ischemia (HI) event, by itself or in combination with other factors as infection (Volpe, 2012). During HI brain blood flow and then oxygen and glucose decreased globally or regionally in the brain. That depletion triggers a continuum of events of cell death, excitotoxicity, inflammation and oxidative stress that will end in tissue damage and different neurological sequelae. The two main pathologies in which HI plays a key role are neonatal hypoxia-ischemia encephalopathy (NHIE) and perinatal arterial ischemic stroke (PAIS).

2.NEUROLOGIC PERINATAL PATHOLOGIES

2.1 Neonatal Hypoxia-Ischemia Encephalopathy

NHIE has an incidence of 2 to 6 per 1000 live births in developed countries (Arnaez et al., 2017; Gale et al., 2017; García-Alix et al., 2009; Hayakawa et al., 2014; Kurinczuk et al., 2010). The

aetiology of NHIE is not always apparent, although intrapartum events often precede the pathology (Martinez-Biarge et al., 2013). Some of them are umbilical cord knots, shoulder dystocia, placental abruption or maternal shock (Abdelaziz et al., 2017; Hayes et al., 2013; Martinez-Biarge et al., 2013). Other relevant antepartum risk factors are gestational age longer than 41 weeks, pre-eclampsia, as well as placental abnormalities and chorioamnionitis (Hayes et al., 2013; Martinez-Biarge et al., 2013).

To diagnose NHIE it is mandatory that perinatal asphyxia was previously detected. International agreement determines that perinatal asphyxia is defined by the presence at birth of one or more of the following: 10-minute Apgar score < 5, prolonged resuscitation (> 10 minutes) after birth, umbilical cord or first neonatal blood gas pH < 7.0, or cord or base deficit > 12 (D'Alton et al., 2014; Parikh and Juul, 2018). In addition, clinical evidences of brain disturbance (encephalopathy) must be apparent: decreased level of consciousness, decreased tone and/or spontaneous activity, decreased primitive reflexes and abnormal autonomic function, abnormalities on amplitude-integrated EEG are taken into account when available to detect encephalopathy (Parikh and Juul, 2018). The diagnosis must be complemented with neuroimaging evidence, being magnetic resonance imaging (MRI) the standard one (D'Alton et al., 2014; Ho et al., 2016; Weeke et al., 2018). Severity of encephalopathy is graded as mild (grade I), moderate (grade II) and severe (grade III) (Sarnat and Sarnat, 1977). While mild cases have almost no sequelae, moderate to severe NHIE develops seizures and other neurological abnormalities soon after the insult (Adhikari and Rao, 2016) and 75% will develop CP in the first years of life with delayed motor, social and language milestone accomplishment (Adhikari and Rao, 2016; Martinez-Biarge et al., 2011). Long-term studies have demonstrated this lifelong impairment with memory, language, sensorimotor and even behavioural deficits present in children from seven to eighteen years old (de Vries and Jongmans, 2010) (Fig.1).

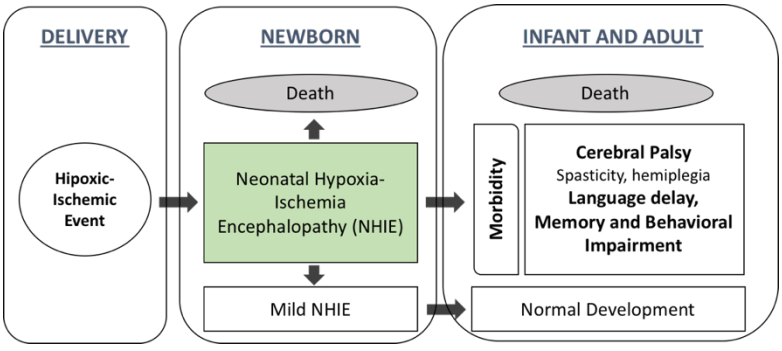


Figure 1. Scheme of NHIE outcome. *Adapted from Lee et al., 2013*

That neurodevelopmental outcome correlated with the intensity and localization of damage as observed in MRI (de Vries and Jongmans, 2010; Martinez-Biarge et al., 2011; Twomey et al., 2010). The classic topography of damage includes parasagittal cerebral cortex, basal ganglia, thalamus and brainstem. Usually,

watershed regions are affected as a consequence of the poor vascularization and regulation of vascular branches. Consistently, after an acute and very severe damage for example as a consequence of placental abruption, peri-rolandic cerebral cortex, paracentral cortex, basal ganglia or thalamus are affected, besides a significant damage of the internal capsule or the cranial nerve motor nuclei in the brainstem (Johnston and Hoon, 2006; Michoulas et al., 2011; Uria-Avellanal and Robertson, 2014; Volpe, 2012). This produces an extrapyramidal pattern of impairment with rigidity and movement disorder (Johnston and Hoon, 2006). Although white matter is less vulnerable than in previous stages of development, it can still be affected, particularly the subcortical cerebral white matter and the overlying grey matter (Johnston and Hoon, 2006; Volpe, 2012). In this case brain damage is associated with a more important cognitive than motor impairment, which is defined by spasticity and upper motor neuron abnormalities (Johnston and Hoon, 2006; Volpe, 2012) and it is usually caused by less intense but longer insult (Johnston and Hoon, 2006; Uria-Avellanal and Robertson, 2014) .

2.2 Perinatal Arterial Ischemic Stroke

PAIS is defined as "a group of heterogeneous conditions in which there is focal disruption of cerebral blood flow secondary to arterial or cerebral venous thrombosis or embolization, between 20 weeks of fetal life through the 28th postnatal day, confirmed by neuroimaging or neuropathological studies" (Raju et al., 2007). PAIS is diagnosed from birth to 28th postnatal day and affects 1/2300 to 1/5000 live newborns (Machado et al., 2015; Raju et al., 2007; Swartz et al., 2017). Although the aetiology remains sometimes unclear, most of the risk factors are shared with NHIE (Machado et al., 2015; Martinez-Biarge et al., 2016; Michoulas et al., 2011; Sreenan et al., 2000). Intrapartum period is considered the one with the highest risk (Martinez-Biarge et al., 2016). Other risk factors are pre-eclampsia, chorioamnionitis, placental abruption, meconium aspiration syndrome or hypoglycaemia (Hayes et al., 2013; Machado et al., 2015; Martinez-Biarge et al., 2016; Raju et al., 2007; Sreenan et al., 2000). However, PAIS specific risk factors are thrombophilia (Kenet et al., 2010), placental abnormalities (Hayes et al., 2013) and congenital heart diseases (Fernández-López et al., 2014). Probably because of the direct vascular route from the cava vein to the left carotid artery through the Ductus Arteriosus (Fernández-López et al., 2014), perinatal arterial ischemic stroke (PAIS) predominantly affects the left hemisphere (Lee et al., 2010; Machado et al., 2015; Michoulas et al., 2011), affecting the left middle cerebral artery territory (Hayward and Adappa, 2014; Sreenan et al., 2000).

Seizures are an important symptom after PAIS, usually in the first 48 hours of life (Billinghurst et al., 2017; Klemme et al., 2017; Lee et al., 2010; Machado et al., 2015; Nelson and Lynch, 2004).

25% of seizing PAIS babies will suffer epilepsy or remote symptomatic seizures afterwards (Billinghurst et al., 2017). Half of the affected newborns develop motor or cognitive impairment as hemiplegia, spasticity or language delay (Lee et al., 2010; Nelson and Lynch, 2004; Sreenan et al., 2000).

2.3 Pathophysiology

As mentioned before, the primary cause of damage in PAIS and NHIE is the depletion of brain blood flow and consequently the levels of glucose and oxygen. In fact, in focal HI damage (stroke) the injury can be divided in two main regions: the core of the insult, where the blood flow decreases until less than 7mL/100g/min when normal flow is 50-60mL/100g/min, and penumbra, with a blood flow between 7 and 17mL/100g/min (Mehta et al., 2007) (Fig. 2A). Such differentiation is less clear in diffuse HI damage, where selective neuronal necrosis is predominant (Volpe, 2012).

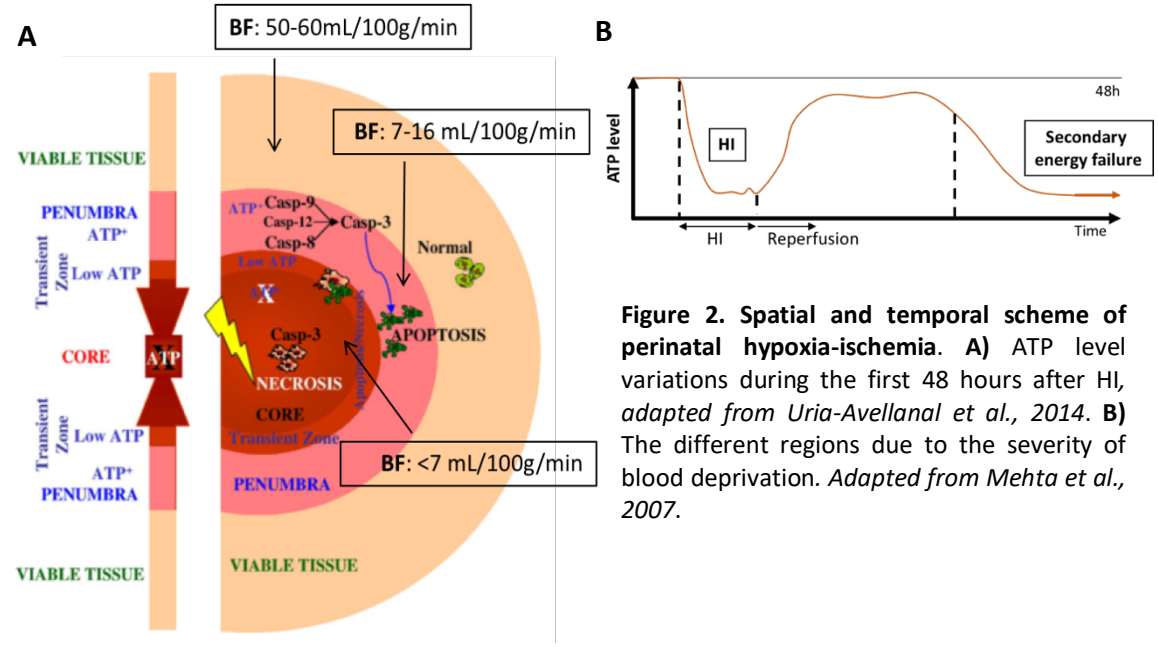


Figure 2. Spatial and temporal scheme of perinatal hypoxia-ischemia. **A)** ATP level variations during the first 48 hours after HI, *adapted from Uria-Avellanal et al., 2014*. **B)** The different regions due to the severity of blood deprivation. *Adapted from Mehta et al., 2007*.

Pathophysiology of NHIE and PAIS is an evolving process. It starts with the decrease of glucose and oxygen leading to a primary energy failure, triggering the anaerobic metabolism (James and Patel, 2014) (Fig. 2B). Due to the anaerobic metabolism fails preventing the reduction of nucleotide triphosphate concentration like adenosine triphosphate (ATP) cannot be prevented (Blumberg et al., 1996; Lorek et al., 1994; Penrice et al., 1997a); also, a severe acidosis, caused by the lactic acid and by proton pumping ATPases impairment occurs (Penrice et al., 1997a; Uria-Avellanal and Robertson, 2014). Finally, blood flow returns to basal condition initiating the

reperfusion phase when pH, metabolites levels and ATP recover their basal levels (Blumberg et al., 1996; James and Patel, 2014; Lorek et al., 1994) (Fig. 2B). However, eventually a second energy failure happens, characterized by a decrease of ATP with no pH changes and increased lactate levels (Fig.2B). This energy impairment is mainly a consequence of a mitochondrial dysfunction and a continuum of oxidative stress, inflammation and excitotoxicity (Blumberg et al., 1996; James and Patel, 2014; Lorek et al., 1994; Northington et al., 2007; Penrice et al., 1997a). Taken together those events lead to a complex situation, in which oxidative stress, inflammation and excitotoxicity feedback potentiates each other in the so-called "deadly-triad", that can last for hours or even days.

Besides the energy depletion per se, this first phase triggers two main pathophysiological events. One is the oedema as a consequence of the ATP-dependent pump impairment like Na^+/K^+ ATPase, responsible for the Na^+/K^+ gradient (Alix, 2006). This induced an important influx of Na^+, Cl^- and water inside the cell producing a cytotoxic oedema (Brekke et al., 2017), observed soon after HI and that last for several days (Mujsce et al., 1990), although last days is mainly vasogenic (Justicia et al., 2008; Siemonsen et al., 2012). The second one is excitotoxicity, caused by a combination of the excitatory aminoacid release, like glutamate, due to membrane polarity failure (Brekke et al., 2017) and the reversal of the glutamate transporter in astrocytes, as a consequence of sodium gradient loss (Johnston, 2005) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKβ) activation (Fatemi et al., 2009) (Fig. 3). High levels of glutamate are observed shortly after HI (Dang et al., 2017; Pazos et al., 2013), and remain elevated for days (Dang et al., 2017; Pazos et al., 2012). In fact, levels of glutamate in cerebrospinal fluid (CSF) correlates with the severity of NHIE (Johnston and Hoon, 2006).

These events lead to a remarkable intracellular calcium increase induced by increased Na^+ influx that revers the Na^+/Ca^{2+} exchange protein (Alix, 2006; Uria-Avellanal and Robertson, 2014) and glutamate activation of glutamate receptor N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptor. Calcium is an important second messenger that play a key role in the pathogenesis of HI promoting cell death and oxidative stress (James and Patel, 2014). On the one hand, high cytosolic calcium concentration actives lipases, proteases or endonucleases that promote cell death (James and Patel, 2014). It also activates calmodulin, which increases the nitric oxide (NO) levels through NO synthase (Thornton et al., 2017b a) and promotes superoxide production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Thornton et al., 2017a b). This superoxide can be transformed into hydrogen peroxide and then to water, by superoxide dismutase and catalase.

Also, it can interact with NO to produce peroxynititre, an effect aggravated by the massive production of NO due to the induction of inducible NO synthase (iNOS) (Martínez-Orgado et al., 2006). Peroxynitrite is highly toxic, promoting lipid peroxidation, protein oxidation and DNA damage (Thornton et al., 2017a b). On the other hand, immature mitochondria are less efficient at buffering calcium, thus its concentration increases rapidly inside the organelle. Calcium and NO will disrupt the electron transport chain, producing reactive oxygen species (ROS), and promoting cell death by apoptosis (James and Patel, 2014; Thornton et al., 2017a, 2017b) (Fig.3).

BOX1: Calcium during a HI event

Calcium precipitates can be observed in the mitochondria of neurons in the core of the insult since the very first 30 minutes after HI (Puka-Sundvall et al., 2000) (Puka-Sundvall *et al.*, 2000 a) as well as in swollen synaptic structures (Puka-Sundvall et al., 2000 a). The first precipitates are observed in penumbra neurons 3 hours after HI. For the next 24 hours, calcium accumulates in other neuronal structures like cytoplasm or endoplasmic reticulum meanwhile cells shows morphologic abnormalities or undergo cell death (Puka-Sundvall et al., 2000 a). Calcium accumulation happens also in astrocytes (Puka-Sundvall et al., 2000), oligodendrocytes and myelin sheats (Micu et al., 2006). It is important to remark that due to its small volume, even small increments of calcium via NMDA receptors could be excitotoxic in oligodendrocyte processes and neuron dendrites (Jantzie et al., 2015). Activation of NMDA receptors during HI has demonstrated to be pro-apoptotic (Fan et al., 2015) and damaging premyelinated axons (Alix and Fern, 2009).

As explained before, the disruption of mitochondrial electron transport systems, NADPH oxidases and NO synthases are one the main sources of oxidative stress in HI. During primary energy failure, ROS and reactive nitrogen species increment inside cells, but it is reperfusion which will boost its production into a high toxicity level (James and Patel, 2014). Microglia is the other main source of ROS and NO (Li et al., 2005; Zhao et al., 2016) (Fig. 3).

HI also produces a prompt inflammation response, being microglia the first agent. Microglia rapidly proliferates and acquires a macrophage-like phenotype, phagocyting cell debris, producing inflammatory and anti-inflammatory cytokines, ROS, NO and metalloproteases (Hellström Erkenstam et al., 2016; Liu and McCullough, 2013). Besides, microglia not only release glutamate thus increasing excitotoxicity (Hagberg et al., 2015), but also expresses functional NMDA receptors (Kaindl et al., 2012). Its activation triggers a long-lasting morphological change

into an active shape, promoting the secretion of numerous cytokines, ROS or NO (Kaindl et al., 2012). Microglia releases a wide array of interleukins (IL) as IL-1α , IL-1β, IL-6, IL-10, IL-12, IL-13; monocyte chemoattractant protein-1 (MCP1) or agonists of death receptors like tumor necrosis factor-related apoptosis induced ligand (TRAIL), tumor necrosis factor alpha (TNFα) or FasL (Fas ligand) (Hagberg et al., 2015; Hellström Erkenstam et al., 2016; Kaindl et al., 2012; Kichev et al., 2014). Other cells like astrocytes or mast cells contribute to inflammation response releasing TNFα, IL-1β, TGF-β1 (transforming growth factor beta-1) or MCP1; both of them being early responders too (Faustino et al., 2011; Hagberg et al., 2015; Jin et al., 2009). Astrocytes are also an important source of TRAIL, which induce oligodendrocyte and neurons death through DR5 receptor (Kichev et al., 2014). Furthermore, this activated and neurotoxic astroglia can be induced by activated microglia through IL-1β, TNFα and the complement molecule C1q (Liddelow et al., 2017) (Fig. 3).

After this innate immune response that can last several days after HI, there is also an adaptive immune response of T Lymphocytes (Liu and McCullough, 2013; Winerdal et al., 2012). Both T-helper and T-cytotoxic cell have been characterized from second week to the third month after HI (Winerdal et al., 2012), or even up to the seven month (Hagberg et al., 2015).

BOX2: Astrocytes and microglia, the other side of the coin.

Although astroglia and microglia contributes actively to inflammation and hypomyelination, it also plays neuroprotective key roles after HI and during development. State-of-the art microglial research has characterized different anti-inflammatory, wound-healing and tissue homeostasis-protectant phenotypes (Liu and McCullough, 2013; Mecha et al., 2016) which would help to reduce the damage.

Furthermore, astrocytes are an important source of glutathione, thioredoxins and other antioxidants defences, provide metabolic support and reuptake glutamate (Brekke et al., 2017; Liu and McCullough, 2013; Romero et al., 2014). They release PDGFRα (platelet derived growth factor receptor α) and other molecules needed for oligodendrocyte progenitor cell (OPC) maturation and short and long term survival (Clemente et al., 2013; Gard et al., 1995; Moore et al., 2011). Because of this protective role, the damage to astrocytes by HI, with their processes disintegrating and its metabolism affected (Alix et al., 2012; Back et al., 2007; Brekke et al., 2017), might be particularly harmful.

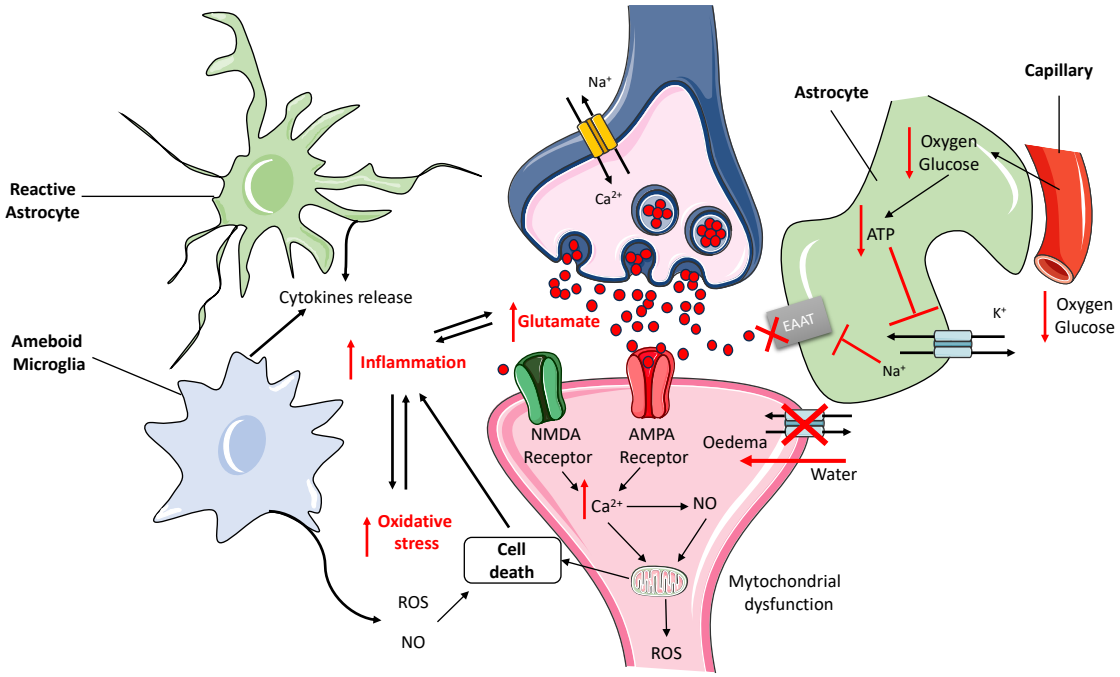


Figure 3. Pathophysiology of a HI/PAIS event. Scheme of the events which followed a HI/PAIS event. The high increase of intracellular calcium induces the release of glutamate, which cannot be re-uptaked by the astrocytes due to the energy deprivation. Therefore, the activation of glutamate receptors potentiates calcium influx, the oxidative stress and eventually the cell death by excitotoxicity. This produces a strong inflammatory response which exacerbates both oxidative stress and excitotoxicity. ROS=reactive oxygen species; NO=nitric oxide; NMDA receptor=N-methyl-D-aspartate receptor, AMPA receptor=α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. *Adapted from Johnston et al., 2007*.

BOX3: Cell death.

Cell death during perinatal brain injury has been characterized as an apoptotic-necrotic continuum, where morphological and biochemical characteristics of both kind of cell death and a hybrid between both can be observed (Northington et al., 2007). It is well know that immature brain is prone to apoptosis (Hagberg et al., 2016), and caspase-3 is involved in cell-death after HI (Manabat et al., 2003; Northington et al., 2007). Necrosis is the main kind of cell death in the core (Carloni et al., 2007). However, several studies have pointed out that the presence of this hybrid apoptosis-necrosis cell death may be consequence of a failed fulfilment of apoptosis due to the energy deprivation (Carloni et al., 2007; Portera-Cailliau et al., 1997). This would explain why the area of necrotic cells increases over time affecting the penumbral area (Carloni et al., 2007; Portera-Cailliau et al., 1997). In NHIE, this "non-controlled death" is, for example, the one caused by cytosolic oedema when the membrane rupture releases molecules known as DAMPs (damage associated molecular patterns) to the medium, triggering the inflammation response (Thornton et al., 2017c).

2.4 Hypomyelination

As explained before, damage induced in white and grey matter after a neonatal HI insult correlated with motor and cognitive impairment that is not repaired by adolescence (de Vries and Jongmans, 2010; Martinez-Biarge et al., 2011) (Fig. 4A).

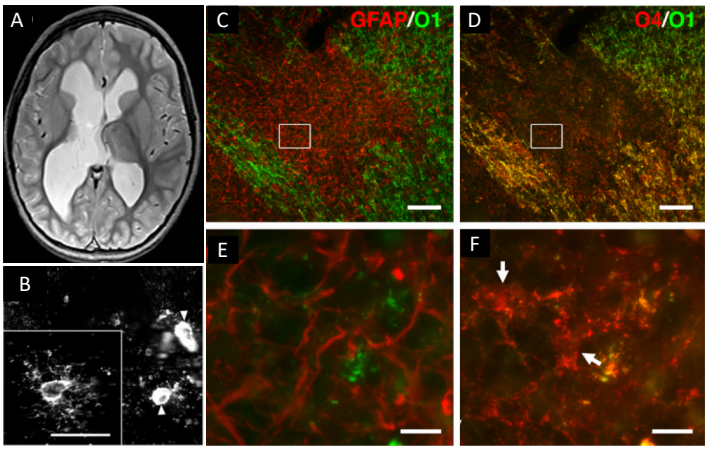


Figure 4. HI induced damage on oligodendrocytes and myelination. **A)** A MRI T2-weighted image which shows diffuse gray and white matter volume loss and dilatation of lateral ventricules; **B)** Pyknotic preOLs 24h after HI *versus* control cell (inset); **C)** and **D)** Gliotic lesion in white matter, where strong astrocytes staining (GFAP) correlated with few immature oligodendrocytes (O1) and a strong preOL signal (O4), showing an arrested maturation stage, one week after ischemia. *Taken from Ho et al., 2016; Back et al., 2002; Riddle et al., 2012*.

After neurons, oligodendrocytes are the most sensitive cell to the "deadly triad"-induced by the HI insult(Back et al., 2002a; Buser et al., 2010; Miller et al., 2007; Rosenberg et al., 2003), particularly the late oligodendrocyte progenitor or preoligodendrocyte (preOL) stage (Fig. 4B). First, excitotoxicity through NMDA and AMPA activation causes both morphology alteration and death in preOL (Follett, 2004; Jantzie et al., 2015; Káradóttir et al., 2005; Salter and Fern, 2005). Second, oxidative stress induces accumulation of lipid peroxidation (Back et al., 2005) and glutathione depletion NMDA-mediated (Rosenberg et al., 2003), which are particularly harmful to those cells, mainly because of its immature antioxidant system (Fragoso et al., 2004). Moreover, microglia and astrocytes can directly damage preOL by activating oxidative stress releasing substances like ROS and peroxynitrite, and releasing cytokines (Deng et al., 2008; J. Li et al., 2008; Li et al., 2005; Miller et al., 2007). Astrogliosis overlapping the lesion days after damage has been widely described (Buser et al., 2012; Segovia et al., 2008). Although oligodendrocyte progenitor cells (OPC) proliferation and maturation is enhanced after HI, they fail to mature, remaining in a preOL stage (Segovia et al., 2008) (Fig. 4C and D). This arrested maturation will eventually lead to hypomyelination that has been studied in humans (Buser et al., 2012), rats (Janowska and Sypecka, 2018; Villapol et al., 2011) and rabbits (Buser et al., 2010). This hypomyelination has been also observed in rat pups after neonatal stroke (Villapol et al., 2011).

Noteworthy, along with hypomyelination axonopathy can also be observed (Alix et al., 2012). Axons are also sensitive to HI damage by glutamate receptor activation and calcium influx (Alix et al., 2012; Back et al., 2007; Micu et al., 2006; Puka-Sundvall et al., 2000; Shen et al., 2012).

2.5 Particularities of immature brain

Immature brain has several characteristics which makes it more sensitive to HI or stroke than the adult brain (Martínez-Orgado et al., 2007). However, neonatal brain has a reduced cerebral blood flow autoregulation which impairs buffering the CBF increase occurring after HI and inducing oxidative stress (Vutskits, 2013). Besides, immature brain is more sensitive to glucose deprivation since it has less glucose transporter and with lower capacity (Brekke et al., 2017).

Neonatal brain is also more susceptible to excitotoxicity (Fatemi et al., 2009). Around last weeks of pregnancy and first postnatal weeks, immature brain is highly excitable due to an over expression of NMDA and AMPA receptor and to the fact that gamma-aminobutyric acid (GABA) receptor is excitatory at that age (Jensen, 2002). Furthermore, both NMDA and AMPA receptor are particularly permeable to calcium (Kirson *et al.*, 1999; Talos *et al.*, 2006 a; Talos *et al.*, 2006 b; Ewald and Cline, 2009) because upregulation of NR2B and NR2D NMDA subunits (Ewald and Cline, 2009; Kirson et al., 1999) and nonGluR2/GluR2 AMPA conformation in rodents (Talos *et al.*, 2006 a) and humans (Talos *et al.*, 2006 b). Its regulation, and therefore sensitivity, its time- and cell- dependent (Jensen, 2002; Talos *et al.*, 2006 a; Talos *et al.*, 2006 b; Kaindl *et al.*, 2012). Furthermore, glutamate and aspartate levels in cerebrospinal fluid correlates with the NHIE severity (Johnston and Hoon, 2006).

Another important characteristic is that immature brain is more sensitive to oxidative stress (Johnston et al., 2011). Since arterial oxygenation saturation in fetal blood is around 40-45% and it is increased to 80-90% after delivery, neonatal brain is already in a pro-oxidative situation (Fatemi et al., 2009). This is aggravated by the lower concentration of antioxidant molecules like vitamin E, β-carotene or melatonin (Gitto et al., 2009); the presence of unsaturated fatty acid (Gitto et al., 2009; Perrone et al., 2015) which potentiates lipid peroxidation and ROS generation; and the developmental regulation of enzymes involved in ROS clearance (Perrone et al., 2015; Shim and Kim, 2013), with less catalase activity and glutathione peroxidase concentration in developing than in mature oligodendrocyte, for example (Baud et al., 2004). Moreover, plasma from healthy term newborns shows higher quantities of free iron due to less iron-binding protein concentrations, which produces hydroxyl radicals by hydrogen peroxide reaction (Buonocore et al., 2001).

Immune response and inflammation are also more harmful for immature than mature brain (Johnston et al., 2011). Besides the fact of a different intracellular signalling of inflammatory pathways as NF-Kβ (Vexler and Yenari, 2009), the profile of immune cell infiltration after brain damage is different in adults and neonates (Vexler and Yenari, 2009; Winerdal et al., 2012). In this context, the integrity of brain blood barrier (BBB) is decisive. Last studies pointed out that BBB is more developed at birth than usually supposed (Moretti et al., 2015) and that its permeability is better preserved after neonatal than after adult stroke (Fernández-López et al., 2012). By contrast, as HI is a more global and diffuse damage, a time-lapse leakage of BBB have been observed after a HI insult (Ek et al., 2015). Although BBB shows higher stability after neonatal stroke, physiological BBB maturation is impaired which could affect developmental angiogenesis (Fernández-López et al., 2013a).

Another possible developmental alteration could arise from the activated state of microglia after the damage. Since microglia have an important role during postnatal development promoting myelination, synaptic pruning and neuronal activity (Low and Ginhoux, 2018), its prolonged activation may alter the appropriate circuitry development.

Overall, these characteristics are aggravated by the fact that during postnatal development different neurons undergo apoptosis (Denaxa et al., 2018; Kim and Sun, 2011; Nikolić et al., 2013). That physiological process is important to prune neuronal networks, eliminating the excess of progenitor cells and neurons, in order to reach an excitatory-inhibitory balance (Denaxa et al., 2018; Kim and Sun, 2011). Not only neuronal activity but also neurotrophins are important to promote or inhibit apoptosis, for which neonatal neurons are predisposed to (Denaxa et al., 2018; Nikolić et al., 2013).

Finally, postnatal brain is known for its plasticity and the synapse formation (Kolb and Gibb, 2011). Moreover, proliferative long-term responses in the subventricular zone and subgranular zone has been described after HI in both ipsilateral and contralateral hemisphere (Brégère et al., 2017; Ong et al., 2005). Although some new immature neurons are observed, particularly in the subgranular zone (Brégère et al., 2017), these HI-induced cells are mainly astrocytes and oligodendrocytes (Ong et al., 2005).

2.6 Neuroprotective Strategies

Despite the high incidence and deleterious consequences of both PAIS and NHIE, there is no other approved treatment than hypothermia for NHIE in term infants (Sagredo et al., 2018).

Newborns with a diagnosis of PAIS are only treated for complications as infection or seizures, with no proper treatment for the stroke itself (Armstrong-Wells and Ferriero, 2014; Cnossen et al., 2009).

Therapeutic hypothermia

Therapeutic hypothermia (TH) is the only treatment currently approved for HI, in which whole body are cooled to 33.5ºC for 72h after HI (Davidson et al., 2015). Use of TH after HI improves survival and reduce disabilities in different clinical and preclinical studies (Fatemi et al., 2009). However, when TH is applied later than 6 hours after HI or the pathology is more severe, hypothermia is not neuroprotective (Davidson et al., 2015; Edwards et al., 2010; Sabir et al., 2012). In fact, TH itself could be harmful for neonates if is prolonged or if lower temperatures are used (Shankaran et al., 2017).

Hereby, there is a need of new synergic molecules which can act with TH, increasing therapeutics windows or improving neuroprotective effects after moderate or severe HI. Other promising molecules are melatonin and erythropoietin. Both melatonin and erythropoietin have been proved to reduce cell death and brain damage in animal models (Lee et al., 2016; Sinha et al., 2018), by mechanisms that include neurogenesis and anti-inflammatory modulation (Blanco et al., 2017; Fan et al., 2011; Lee et al., 2016; Sinha et al., 2018; Wu et al., 2016).

In conclusion, due to the high incidence and deleterious consequences of both NHIE and PAIS and the partial results of TH in NHIE, the research of new molecules which administrated with hypothermia could help to improve the pathology outcome is one of the first need of current paediatric research. The multifactorial complexity of pathologic events prompt the necessity of a pleiotropic molecule, which can act at many different levels. In this context, cannabinoids have risen as interesting therapeutic compounds (Fernández-Ruiz et al., 2015; Martínez-Orgado et al., 2007; Sagredo et al., 2018).

3. CANNABINOIDS

Cannabis sativa is a domesticated hemp whose medical use has been explored worldwide for centuries, from ancient China to Assyria or Persia (Ligresti et al., 2016). “Cannabinoid” is the traditional name given to a group of terpenophenolic compounds mainly found in the *C. sativa* plant (Andre et al., 2016). However, since the description of the Endocannabinoid System (ECS), the term cannabinoid term includes: 1) phytocannabinoids, the ones produced by the plant; 2) endocannabinoids, the cannabinoids synthesized by vertebrate animals and 3) synthetic

cannabinoids, which have been chemically synthesized in the laboratory (Andre et al., 2016; Ligresti et al., 2016).

3.1 The Endocannabinoid System

Research on phytocannabinoids in the XX century led to the discovery of ECS (Ligresti et al., 2016). The ECS is an endogenous system with complex interaction with other systems, like the endocrine or the immune system, playing important physiological roles such as neurotransmission modulation. ECS consists of ligands, the endocannabinoids, the synthesis and degradation enzymes and the receptors (Table 1).

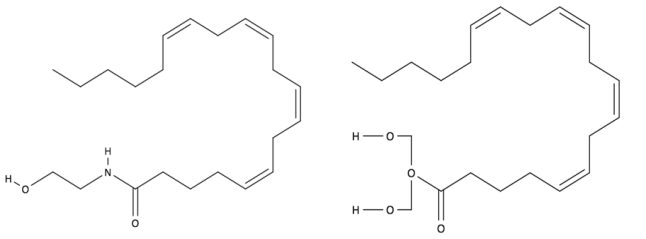
The Endocannabinoid System	
Endocannabinoids	2-arachidonoylglycerol (2-AG); *N*-arachidonoylethanolamine (AEA)
Synthesis enzymes	Diacylglycerol-lipase (DAGL); N-arachidonoyl phosphatidylethanolamine (NAPE)
Degradation enzymes	Monoacylglycerol lipase (MAGL); Fatty acid amide hydrolase (FAAH)
Receptors	Cannabinoid Receptor 1 (CB_1); Cannabinoid Receptor 2 (CB_2)

Table 1. Main components of the Endocannabinoid System

3.1.1 Endocannabinoids

Defined as small lipids-derived signalling molecules, endocannabinoids can be found throughout the human body, including peripheral and central nervous system (Augustin and Lovinger, 2018). The two main endocannabinoids are 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995) and *N*-arachidonoylethanolamine or anandamide (AEA) (Devane et al., 1992) (Fig. 5).

2-AG was discovered in 1995 (Mechoulam et al., 1995; Sugiura et al., 1995) and it is a full agonist of cannabinoid receptor 1 (CB_1) and 2 (CB_2) (Stella et al., 1997; Zou and Kumar, 2018). At least in one order of magnitude more abundant than AEA (Stella et al., 1997; Sugiura et al., 1995), 2-AG is produced



Anandamide (AEA) 2-Arachidonoyl glicerol (2-AG)

Figure 5. Chemical structures of *N*-arachionoylethanolamine or anandamide (AEA) and 2-arachidonoylglycerol (2-AG).

constitutively at basal conditions (Hashimotodani et al., 2007; Lee et al., 2015). On the other hand, AEA is a partial agonist of CB_1 with non-effect on CB_2 receptor (Silva et al., 2013; Zou and Kumar, 2018). AEA acts also as an agonist of transient receptor potential type V 1 (TRPV1) (Fenwick et al., 2017).

3.1.2 Synthesis machinery

Endocannabinoids are synthesized "on demand" after an increase of intracellular calcium and/or in presence of substrate (Gregg et al., 2012; Hashimotodani et al., 2013; Shonesy et al., 2015) and released with a mechanism that remains unknown (Nicolussi and Gertsch, 2015).

2-AG is mainly synthesized by diacylglycerol-lipase (DAGL) from diacylglycerol (Shonesy et al., 2015; Tanimura et al., 2010). Although there are two isoforms, DAGLα and DAGLβ (Blankman and Cravatt, 2013), only DAGLα is expressed in brain, localized in postsynaptic neurons (Gregg et al., 2012), astrocytes and microglia (Suárez et al., 2011).

AEA synthesis is far more complex than 2-AG's with at least four pathways involved in its production (Blankman and Cravatt, 2013). The principal substrate is N-arachidonoyl phosphatidylethanolamine (NAPE) which is directly cleaved by NAPE-phospholipase D (NAPE-PLD) to AEA (Di Marzo et al., 1994; Leishman et al., 2016). NAPE-PLD is expressed in post-synaptic terminals (Hegyi et al., 2012; Reguero et al., 2014) and in micro and astroglia processes (Hegyi et al., 2012) .

3.1.3 Degradation machinery

After an endocannabinoid has exerted its function, it is rapidly re-uptaken into the cell to be degraded. The re-uptake mechanism is not full elucidated but it may include binding to highly caveolae-rich membrane domains (Nicolussi and Gertsch, 2015) or passive diffusion through membrane (Kaczocha et al., 2009; Nicolussi and Gertsch, 2015). Some studies have pointed out the existence of intracellular carrier like fatty acid binding proteins (FABPs) which will transport either AEA and other cannabinoids like cannabidiol (CBD) (Kaczocha et al., 2012) to other intracellular targets such as peroxisome proliferator-activated receptors (PPAR) (Kaczocha et al., 2012; Yu et al., 2014) or endocannabinoids degradation enzymes (Kaczocha et al., 2009; Nicolussi and Gertsch, 2015) (Fig. 6).

Fatty acid amide hydrolase (FAAH) is an integral-membrane protein, which metabolize AEA to arachidonic acid and ethanolamide (Zou and Kumar, 2018). In brain FAAH expression is mainly

localized on dendrites and dendritic spines of postsynaptic neurons, correlating with presynaptic CB_1 receptor (Baggelaar et al., 2017; Gulyas et al., 2004).

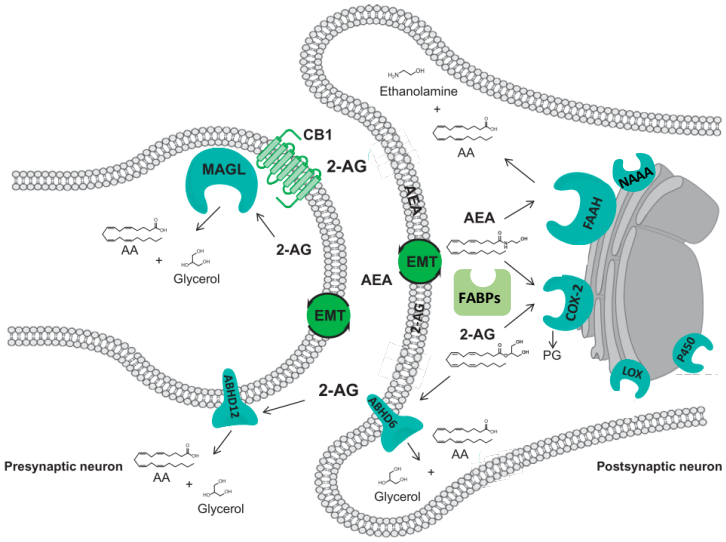


Figure 6. Transport and degradation machinery of the endocannabinoids AEA and 2-AG. Whether by diffusion or by the membrane transporter activity, the endocannabinoids are introduced into the cell, possibly transported by FABP and then degraded by different enzymes. From left to right: AA=arachidonic acid; MAGL= monoacylglycerol lipase; 2-AG=2-arachidonoyl glycerol; ABHD6/12=α/β-hydrolase domain contain 6/12; CB1=cannabinoid receptor 1; AEA= *N*-arachidonoylethanolamine or anandamide; EMT=putative endocannabinoid membrane transporter; FABP= fatty acid binding protein; FAAH=fatty acid amide hydrolase; COX-2=cyclooxygenase 2; LOX=lipoxigenase; NAAA= NAE-hydrolyzing acidamidase; P450= cytochrome P450. *Adapted from Nicolussi et al., 2015.*

2-AG hydrolysis to arachidonic acid and glycerol is mainly carried out by monoacylglycerol lipase (MAGL) (Dinh et al., 2002). MAGL is found presynaptically in axon terminals of cerebral along the brain, matching presynaptic CB_1 expression (Baggelaar et al., 2017; Dinh et al., 2002; Gulyas et al., 2004). Other two enzymes, α/β-hydrolase domain contain 6 (ABHD6) and 12 (ABHD12) are also able of metabolize 2-AG (Navia-Paldanius et al., 2012).

Several enzymes like lipoxygenases, NAE-hydrolyzing acidamidase (NAAA), cytochrome P450, and the cyclooxygenase 2 (COX2) may degrade AEA or 2-AG, producing some endogenous metabolites like prostaglandins (Blankman and Cravatt, 2013; Gómez-Ruiz et al., 2007; Nicolussi and Gertsch, 2015)

3.1.4. Cannabinoid Receptors

3.1.4.a Cannabinoid Receptor 1

CB_1 receptor is a G protein-coupled receptor (GPCR)(Hua et al., 2016; Matsuda et al., 1990), first discovered and cloned in rat and human brain (Devane et al., 1988; Gerard et al., 1990; Gérard et al., 1991; Matsuda et al., 1990). Its structure has been recently characterized (Hua et al., 2016). CB_1 is considered the most abundant receptor protein of the GPCR family in the brain,

widely expressed in both central and peripheral nervous system (Freundt-Revilla et al., 2017; Howlett et al., 2002; Zou and Kumar, 2018). Its localization is mainly presynaptic, in axon terminals of both GABAergic and glutamatergic neurons (Gulyas et al., 2004; Howlett et al., 2002). It has been involved in long-term depolarization and depolarization-induced suppression of inhibition or excitation and consequently memory and plasticity phenomenon (Augustin and Lovinger, 2018; Ohno-Shosaku and Kano, 2014; Oliveira da Cruz et al., 2016; Tanimura et al., 2010; Wang et al., 2018). Outside the nervous system, CB_1 is expressed in liver (Dai et al., 2017), intestines (Wright et al., 2005), muscles (Mendizabal-Zubiaga et al., 2016), adipocytes (Lee et al., 2009) or testis (Gérard et al., 1991) among others.

3.1.4.b Cannabinoid Receptor 2

CB_2 has been considered the "peripheral" cannabinoid receptor since its discovery in 1993 (Munro et al., 1993). CB_2 is a GPCR receptor mainly expressed in immune tissues, like tonsils or spleen (Galiègue et al., 1995; Munro et al., 1993), and immune cells like natural killers cells and macrophages (Galiègue et al., 1995; Turcotte et al., 2016). However, in the nervous system, CB_2 has been well characterized in microglial cells (Núñez et al., 2004) and astrocytes (Fernández-Trapero et al., 2017a), where it is up-regulated in activated states (Fernández-Trapero et al., 2017a; López et al., 2018; Maresz et al., 2005; Turcotte et al., 2016). CB_2 presence in neurons is controversial; several studies have not found expression in the brain (Galiègue et al., 1995; Munro et al., 1993) while others have found it in brain (Callén et al., 2012; Onaivi et al., 2006), in neuronal or endothelial cells in physiological conditions (Brusco et al., 2008; Sickle et al., 2005). Furthermore, several studies have proved that brain CB_2 receptor is up-regulated after stress triggers (Robertson et al., 2017) or other pathological states (Benito et al., 2008; Fernández-Trapero et al., 2017a; López et al., 2018). On the other hand, CB_2 receptor could be responsible for a specific plasticity in hippocampus (Stempel et al., 2016) as well as neurogenesis in subventricular zone (Goncalves et al., 2008; Palazuelos et al., 2012). Its presence and role has been also characterized in the gastrointestinal tract (Wright et al., 2005), liver (Dai et al., 2017), reproductive (El-Talatini et al., 2009) or cardiovascular (Galiègue et al., 1995) system, among others (Zou and Kumar, 2018). As other GPCRs, CB_2 forms heteromers with CB_1 (Callén et al.,

2012), GPR55 (Balenga et al., 2014) and chemokine receptor CXCR4 (Coke et al., 2016) have been found.

3.1.4.c. Non-CB_1 Non-CB_2 Receptors

Cannabinoid ligands are known to interact with many other systems and receptors. GPR55 is a seven membrane coupled to a protein G receptor that was discovered in 1999 (Sawzdargo et al., 1999). GPR55 is found in rat and human brain (Sawzdargo et al., 1999; Sharir and Abood, 2010) as well as in non-nervous tissues like lung, liver or bladder (Sharir and Abood, 2010). Although the exact pathways involved in GPR55 action is still under debate, it seems that both G_q and $G_{\alpha\ 11/12}$ may be involved (Lauckner et al., 2008; Sharir and Abood, 2010). Both CB_1 and CB_2 are able to form an heteromer with GPR55, attenuating GPR55 signalling pathways (Balenga et al., 2014; Kargl et al., 2012).

BOX4: CB_2 agonism as treatment for neurodegerative diseases.

The direct agonism of CB_2 has been largely used as a target for the treatment of both acute and chronic neurodegenerative pathologies (Fernández-Ruiz et al., 2007)

A CB_2 up-regulation has been observed in diverse animal models of chronic neurodegenerative diseases such as Huntington (Sagredo et al., 2009), sclerosis lateral amyotrophic (Espejo-Porras et al., 2018), Alzheimer (López et al., 2018) or Parkinson (Concannon et al., 2016). The use of CB_2 agonism has been able to increment neuron survival (Espejo-Porras et al., 2018; Oddi et al., 2012; M. T. Viscomi et al., 2009) and improve neurological outcome (Espejo-Porras et al., 2018; M. T. Viscomi et al., 2009). CB_2 has been found in glial cells, where its activation seem to decrease of glia activation, associated with neuronal survival (Espejo-Porras et al., 2018; Fernández-Ruiz et al., 2007; López et al., 2018; Sagredo et al., 2009; Tolón et al., 2009). CB_2 agonism also blocks the augmentation of iNOS expression and activity and reduces ROS and peroxynitrites levels (Oddi et al., 2012).

Moreover, the administration of a CB_2 agonist prevented the motor and cognitive impairment associated to a subarachnoid haemorrhage in adult rats, reducing the oedema and BBB disruption induced by this pathology (Fujii et al., 2014b). More remarkably for this thesis topic similar results were obtained in a model of germinal matrix haemorrhage, a pre-term disease, treated with the CB_2 agonist JWH-133 (Tao et al., 2015).

Other important target receptor for cannabinoids is TRPV1. TRPV1 is a channel receptor with six transmembrane domains (De Petrocellis and Di Marzo, 2005) which plays a key role in pain modulation in sensorial neurons (Bisogno et al., 2001; Gómez-Ruiz et al., 2007; Lee et al., 2015). Different endocannabinoids as AEA or phytocannabinoids as CBD are able to activate this receptor (Bisogno et al., 2001; Kano et al., 2009; Marinelli et al., 2007).

Finally, many receptors have been seen to interact with cannabinoids like PPAR receptors (Kaczocha et al., 2012; Yu et al., 2014) or several orphan GPCRs as GPR18 (Morales and Reggio, 2017) as well as other receptors like serotonin (Russo et al., 2005) or adenosine (Morales and Reggio, 2017) receptors. This shows the complex pharmacology of this endogenous system as well as of either synthetic and pharmacological compounds.

3.1.5 Signalling of Cannabinoid Receptors

Broadly, the signalling pathways activated by CB_1/CB_2 receptor depend on the type of cell and G protein coupled to the receptor. Usually both CB_1 and CB_2 receptors are coupled to $G_{i/0}$ protein, which supresses the adenylate cyclase and consequently reduces the cAMP (cyclic adenosine monophosphate)(Bouaboula et al., 1996), the protein kinase A (PKA) activation and calcium influx to the cell (Kim et al., 2005). The cannabinoid receptor activation also induces the activation of ERK1/2 and MAPK pathway (Bouaboula et al., 1996; Hytti et al., 2017), as well as the activation of PI3K/Akt/mTORC1 pathway (Blázquez et al., 2015; Kim et al., 2005; Palazuelos et al., 2012) which is highly involved in the protective and neuroproliferative effect of these receptors (Blázquez et al., 2015; Palazuelos et al., 2012). On the other hand, CB_1 also binds to G_s, increasing cAMP, and G_{q11} (Gómez-Ruiz et al., 2007; Zou and Kumar, 2018). The activation of this receptor may increase the cytosolic calcium from endoplasmic reticulum, via phospholipase C and 1,4,5-triphosphate (IP_3) (Redmond et al., 2016; Zoratti et al., 2003). That mechanism plays an important role in the release of glutamate from astrocytes (Oliveira da Cruz et al., 2016) (Fig. 7).

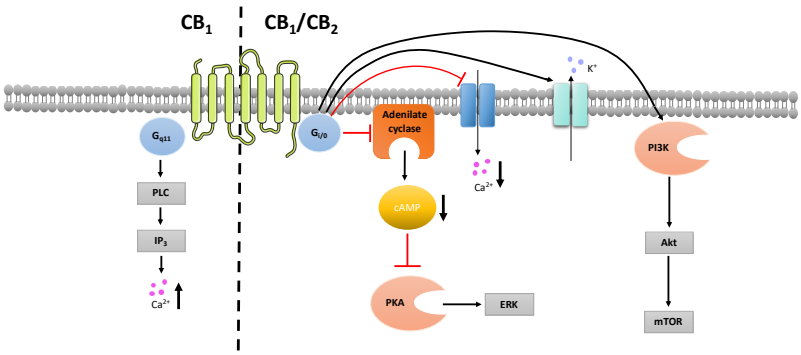


Figure 7.Signalling of cannabinoid receptors. Depending on the alpha subunit associated to the cannabinoid receptor, its activation would induce an specific signalling pathways, explaining the complex and diverse response associated to these receptors. CB_1/CB_2=cannabinoid receptor 1/2; PLC=phospholipase C; IP_3=1,4,5- triphosphate; cAMP= cyclic adenosine monophosphate; PKA=protein kinase A; ERK=extracellular signal–regulated kinases; PI3K=phosphoinositide-3-kinase; mTOR=mammalian target of rapamycin.

Moreover, CB_1 modulates the inhibition of N-type and P/Q-type calcium channel and the activation of rectifying potassium channel via $G_{i/0}$ (Guo and Ikeda, 2004) (Fig. 7).

3.1.6. Physiologic roles of the Endocannabinoid System

One of the main functions of the ECS in the Central Nervous System is the modulation of synaptic transmission, mainly by retrograde modulation in which the endocannabinoids released by the postsynaptic neuron after synapse activates the presynaptic CB_1 receptor. This activation triggers the hyperpolarization of the neuron and consequently ceases the neurotransmitter secretion in both glutamatergic and GABAergic pathways (Augustin and Lovinger, 2018; Haj-Dahmane et al., 2018; Hashimotodani et al., 2013; Ohno-Shosaku and Kano, 2014; Yu et al., 2014). This mechanism participates in several mechanism of plasticity (Fenwick et al., 2017; Lee et al., 2015) and neural function (Augustin and Lovinger, 2018). On the other hand, astrogial CB_1 modulates homo- and hetero-synapses (Oliveira da Cruz et al., 2016).

On the other hand, ECS plays a key role in energy and metabolism regulation (Bénard et al., 2012; Hillard, 2018; Mendizabal-Zubiaga et al., 2016) and in cardiovascular (García et al., 2009; Ho et al., 2008) or endocrine system modulation (Dobovišek et al., 2016), among others. For the topic of this dissertation, is also particularly important the modulation role of ECS in the immune system (Castaneda et al., 2017; Lou et al., 2018).

BOX5: Endocannabinoid modulation of oligodendrocyte maturation.

The endocannabinoid system has been reported to modulate oligodendrocyte proliferation and maturation by the endocannabinoid 2-AG and receptors CB_1 and CB_2 (Gomez et al., 2015). Pharmacological approximations have demonstrated that the inhibition of DAGL, or CB_1 or CB_2 antagonism decreased myelin production, both *in vitro* and *in vivo* (Arevalo-Martin et al., 2007; Gomez et al., 2010). What is more, cannabinoid receptors as well as endocannabinoid synthesis/degradation machinery are modulated during oligodendrocyte maturation, with a peak of DAGL expression in oligodendrocyte progenitors (Gomez et al., 2010). In fact, a recent study has demonstrated that the inhibition of MAGL, main hydrolytic enzyme of 2-AG, is able to reduce oligodendrocyte death by mild activation of AMPA receptors *in vitro* (Bernal-Chico et al., 2015).

3.2 Synthetic cannabinoids

The name of synthetic cannabinoids references to all the compounds chemically synthesized in a laboratory and that pharmacologically interacts with the ECS.

3.2.1 Agonist Ligands

Agonist ligands can be classified attending its chemical structure:

1) Classical cannabinoids englobe all the dibenzopyran derivatives produced by *C. sativa* and its synthetic analogues. Some of the most important are Δ^9- tetrahydrocannabinol (THC), the main component of the plant (Gaoni and Mechoulam, 1964) and a partial agonist of both CB_1 and CB_2 receptor, HU-210 or JWH-133 (Howlett et al., 2002; Pertwee et al., 2010; Soethoudt et al., 2017).
2) Non-classical cannabinoids eliminate the pyran ring of THC structure. Two useful synthetic non-classical cannabinoids are: CP-55940, a potent CB_1/CB_2 agonist, and HU-308, a highly selective CB_2 receptor agonist (Howlett et al., 2002; Pertwee, 2009; Soethoudt et al., 2017) (Fig. 8). HU-308 is highly used in scientific research due to its high specific preference for CB_2 receptor and a well-balanced ligands activation of the different CB_2-associated pathways (Soethoudt et al., 2017). However, HU-308 also binds some off-target receptor as TRPV1 or the dopamine transporter (Soethoudt et al., 2017).

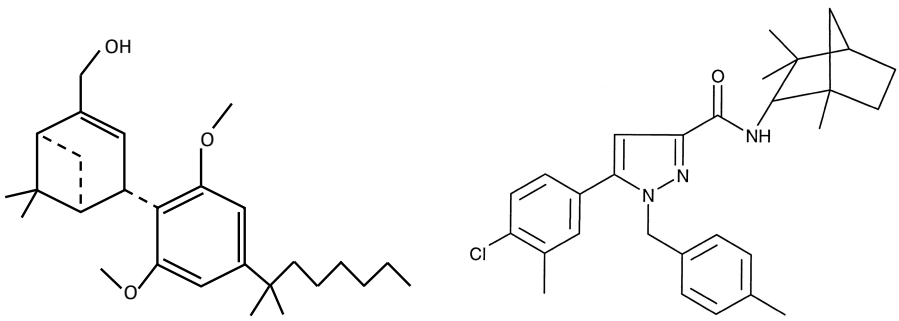
3) Aminoalkylindoles are markedly different from previous cannabinoids since they are derivatives of pravadoline. The main cannabinoid is WIN55212, a potent full CB_1/CB_2 agonist (Howlett et al., 2002).

The fourth chemical structure of cannabinoids are the eicosanoids, which are exclusively represented by endocannabinoids as AEA or 2-AG (Howlett et al., 2002; Pertwee et al., 2010).

3.2.2 Antagonist Ligands

There are two main types of cannabinoid antagonists: diarylpyrazoles, like SR141716A – an specific CB_1 antagonist (Soethoudt et al., 2017)- , or other chemical series, like 6-ioprovadalina or AM630. AM630 and SR144528 are both selective CB_2 antagonist (Pertwee et al., 2010; Soethoudt et al., 2017) (Fig. 8), even though AM630 is also a full agonist of transient-receptor-

potential-related A1 (TRPA1) (Soethoudt et al., 2017). However, these antagonists can also act as inverse agonists, triggering a signal pathway when administered (Howlett et al., 2002).



HU-308 SR144528

Figure 8. Chemical structures of CB_2 agonist HU-308 and CB_2 antagonist SR144628 (SR2), *taken from Rinaldi-Carmona, 1998*.

3.3 Phytocannabinoids

These cannabinoids are terpenophenolic molecules extracted from the plant *Cannabis sativa* (Iuvone et al., 2009; Pertwee, 2008) (Fig. 9). As it has been previously explained, this field research started with the discovery of CBD and THC. THC is the main component of the plant, and main responsible for its psychotropic effects (Gaoni and Mechoulam, 1964) and the well-known tetrad effect of cannabis: hypolocomotion, hypothermia, anhedonia and antinoception (Metna-Laurent et al., 2017; Pertwee, 2008). It is a classical cannabinoid which binds of both CB_1 and CB_2 receptors (Howlett et al., 2002; Pertwee, 2008), with slightly preference for CB_1 (Howlett et al., 2002; Pertwee et al., 2010); the principal responsible for its activities (Blázquez et al., 2015; Howlett et al., 2002; Thapa et al., 2018).

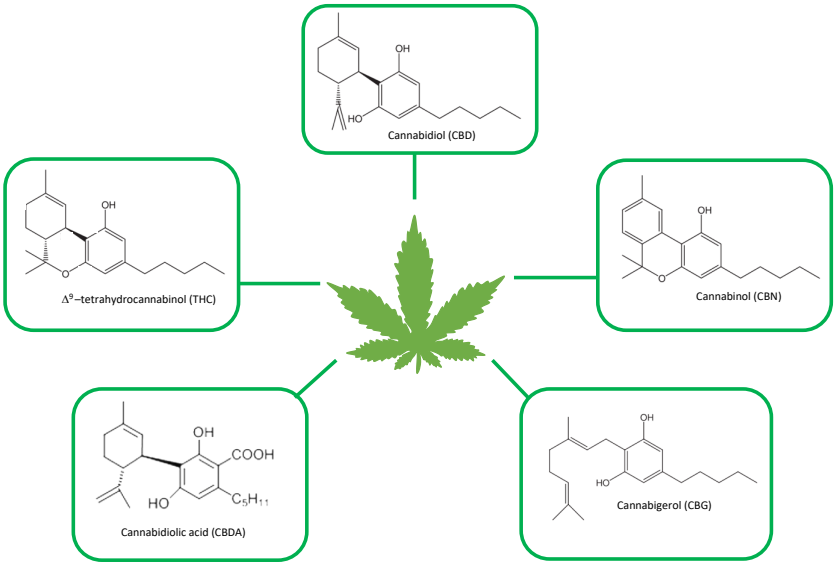


Figure 9. The structures of the phytocannabinoids THC, CBD, cannabidiolic acid, cannabigerol and cannabinol from *Cannabis sativa*. *Taken from Pertwee et al., 2010.*

3.3.1 Cannabidiol

This phytocannabinoid was characterized in 1963 by Mechoulam and Shuo (Mechoulam and Shvo, 1963) and is the second major cannabinoid in the plant, modulating collateral effects of THC (Pisanti et al., 2017). Due to its lipophilic nature, CBD has poor bioavailability but easily crosses the brain blood barrier, and it is lately inactivated by cytochrome P450 (Pisanti et al., 2017).

CBD has a complex pharmacology. It has micromolar affinity for CB_1 and CB_2 receptor (McPartland et al., 2014). Recent studies have proved that can act as allosteric modulator of both receptors (Laprairie et al., 2015; Martínez-Pinilla et al., 2017). In fact, several studies have proved that both CB_1 and CB_2 mediated CBD action (McPartland et al., 2014; Stanley et al., 2015) whereas CBD prevents both AEA re-uptake and hydrolysis (Bisogno et al., 2001). CBD is a TRPV1 agonist (Bisogno et al., 2001; Costa et al., 2004; Hassan et al., 2014; Sagredo et al., 2007) and binds to serotonin receptor 5-HT_{1A} and several PPAR receptors (Hegde et al., 2015; Hind et al., 2016; Pazos et al., 2013; Russo et al., 2005). Moreover, CBD interacts with adenosine receptors (Castillo et al., 2010; Sagredo et al., 2007) .

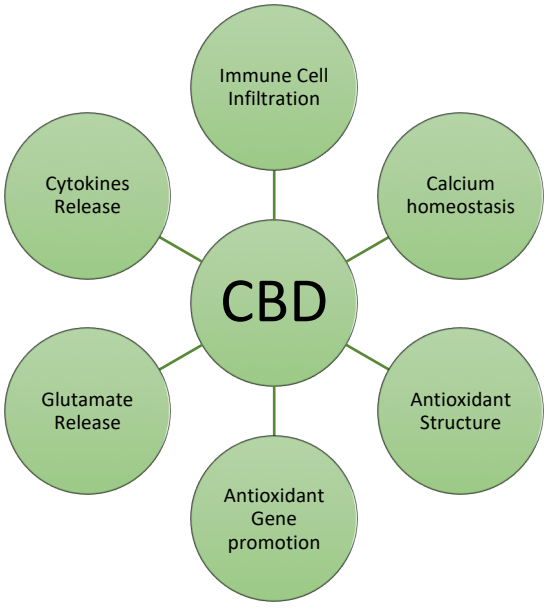


Figure 10. Neuroprotective effects of CBD.

On the other hand, by its own structure with the phenol ring and alcohol group, CBD is a potent antioxidant, more potent even than ascorbate (Hampson et al., 1998). Indeed, decreased levels of reactive oxygen/nitrogen species has been observed after CBD administration (El-Remessy et al., 2003; Iuvone et al., 2004; Mukhopadhyay et al., 2011). Furthermore, CBD is also able of modulate intracellular calcium (Hassan et al., 2014; Iuvone et al., 2004; Mato et al., 2010; McPartland et al., 2014) and reduce glutamate release (Castillo et al., 2010; Gobira et al., 2015; Lafuente et al., 2016). CBD is also a strong immunomodulator, decreasing infiltration (Li et al., 2018), microglia activation (Mecha et

al., 2013) and cytokines and chemokines release (Esposito et al., 2007; Mecha et al., 2013; Vilela et al., 2017) (Fig. 10).

CBD administration is protective in models of different diseases in which inflammation plays a key role, as multiple sclerosis (Mecha et al., 2013), hepatic ischemia(Mukhopadhyay et al., 2011) or Alzheimer (Esposito et al., 2007). Moreover, CBD treatment has showed myelin protective effects, preventing OPC death by both inflammatory and oxidative stress *in vitro* (Mecha et al., 2012). In different animal models of demyelinating diseases CBD by itself or co-administrated with other cannabinoids improves the neurobehavioral outcome diminishing demyelination severity and axonal damage by modulating inflammation (Feliú et al., 2015; Giacoppo et al., 2015; Rahimi et al., 2015). CBD has also demonstrated being a potent anticonvulsivant drug (Gobira et al., 2015; Vilela et al., 2017), culminating with the approval of cannabidiol (Epidiolex ©), for refractory epilepsies and with remarkable results in Dravet syndrome (Kaplan et al., 2017; Porter and Jacobson, 2013)

4. THE ENDOCANNABINOID SYSTEM IN HYPOXIC-ISCHEMIC BRAIN DAMAGE

The ECS is affected by the hypoxia-ischemia insults. Firstly, the levels of AEA, 2-AG and endocannabinoids-like molecules, oleoylethanolamide and palmitoylethanolamide, increase shortly after the damage in adult (Hillard, 2008) and newborn animals (Pazos et al., 2013). Due to AEA and oleoylethanolamide activity in BBB permeabilization (Hind et al., 2015) they might play an important role after HI/PAIS insult. On the other hand, an increase of CB_2 but not CB_1 receptor has been observed in newborn rats in the subventricular zone 48 hours after the insult, which is normalized one week after damage (Fernández-López et al., 2010). Interestingly, a rapid increase of CB_1 but not CB_2 has been observed in an oxygen and glucose deprivation *in vitro* model (OGD) (Fernández-López et al., 2006).

ECS alteration has also been studied in an adult mice model of stroke, where a rapid increase of CB_1 and slower augmentation of CB_2 levels, after an initial decrease, is observed (Zhang et al., 2008). Controversially, another study has proved that CB_1 receptor is decreased in both cortex and striatum 24 hours after middle cerebral artery occlusion in adult mice (Hayakawa et al., 2007b).

5. CANNABINOIDS AS TREATMENT IN NEONATAL HYPOXIA-ISCHEMIA ENCEPHALOPATHY AND PERINATAL ARTERIAL ISCHEMIC STROKE

Several pharmacological ECS modulation has been studied in several animal models of NHIE/PAIS treatment. On one hand, the administration of endocannabinoids such as AEA or 2-AG after neonatal HI in rats, reduces damage and cell death one week after damage by modulating intracellular calcium and decreasing ROS production (Lara-Celador et al., 2012). Endocannabinoid-like oleylethanolamide administration diminishes BBB permeabilization after OGD (Hind et al., 2015).

The agonism of CB_1 and CB_2 receptors is neuroprotective both *in vitro* and *in vivo*. The administration of WIN55212-2after HI insult in newborn rat pups reduces brain volume damage as well as cell death (Fernández-López et al., 2007), which correlates with *in vitro* results where WIN55212-2 also prevents the increase on glutamate, iNOS and TNFα (Fernández-López et al., 2006). Moreover, WIN55212-2 also induces myelin restoration and the increase of OPC proliferation and maturation (Fernández-López et al., 2010). However, different studies have proved that over-activation of CB_1 receptor in the perinatal period could be harmful, since neonatal brain is more susceptible to neuronal death induced by CB_1 (Downer et al., 2007). CB_1 over-activation in immature brain also induces long-lasting T cell development impairment (Lombard et al., 2011) and alters cell adhesion molecules in neurons involved in brain development (Gómez et al., 2007).

In the last years CBD has been pointed out as a good treatment for infant pathologies because it does not bind CB_1 receptors (McPartland et al., 2014). In HI animal models, CBD administration post-insult is associated with a neurophysiological and neurobehavioral improvement (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2013, 2012), in both short (Alvarez et al., 2008; Lafuente et al., 2011) and long-term studies (Pazos et al., 2012). This CBD protection is associated with decreased cell death *in vitro* (Castillo et al., 2010) and *in vivo*, in both piglets (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2013) and rat pups (Pazos et al., 2012). Such effects are related with the reduction of excitotoxicity, neuroinflammation and oxidative stress (Alvarez et al., 2008; Castillo et al., 2010; Pazos et al., 2013, 2012). Finally, CBD is able to prevent the increase of BBB permeability associated to OGD in an *in vitro* study (Hind et al., 2016).

CBD has also been used for stroke treatment in adult mice, where it reduces the infarct volume via serotonin receptor 5-HT_{1A} activation (Mishima et al., 2005) but not via TRPV1 (Mishima et al., 2005) or CB_1 (Hayakawa et al., 2007a, 2004). This neuroprotection is correlated with inflammation decrease (Hayakawa et al., 2007a; Khaksar and Bigdeli, 2017).

Hypothesis and Goals

As described in the Introduction, nowadays there is an urgent need for additional treatments to hypothermia for neonatal encephalopathies. Its complex and multifactorial pathophysiology requires pleiotropic molecules, such as cannabinoids, which can act on different processes involved in the damage. In this context, CBD has been pointed out as a promising drug for neonatal hypoxic-ischemic brain damage. This phytocannabinoid decreases excitotoxicity, oxidative stress and inflammation, i.e. the "deadly triad" in a hypoxic-ischemic event. In fact, very exciting results have been reported in previous works. Furthermore, this compound has no psychotropic activity which allows its safe use in newborns.

HYPOTHESIS

CBD administration post-injury will exert short- and long-term protective effects in models of either neonatal global or focal HI, i.e. NHIE or PAIS respectively. CBD will exert its protective effects by inhibiting the "deadly triad" independently from the global or focal nature of the insult and consequently, protect oligodendrocytes and neurons and modulate microglia and astroglia activation, in a manner in which CB_2 receptor activation would be involved.

GOALS

1. To characterize short- and long-term CBD neuroprotective effects in a focal model of hypoxic-ischemic brain injury in newborn rats.

2. To analyze HI-induced long-lasting hypomyelination in White and Grey Matter and its prevention by CBD.

3. To assess whether CBD hypomyelination prevention is related with brain cell proliferation activation and glial response modulation.

4. To study the involvement of CB_2 receptor activation in CBD protective effects in a newborn rat model of hypoxic-ischemic brain damage.

Methods

1. ANIMAL MODELS

All the animal experimental procedures were previously approved by the Ethical Committee for Animal Welfare of the Hospital Universitario Puerta de Hierro Majadahonda and Hospital Clínico San Carlos (Madrid, Spain), and met the European and Spanish regulation (86/609/EEC and RD 1201/2005, and 2010/63/EU and RD 53/2013).

Two models were used to study the neuroprotective effect of CBD on NHIE. Global HI was reproduced using the Rice-Vannucci procedure, from now on named just as HI, while temporary middle cerebral artery occlusion (MCAO) protocol was used for PAIS. Both models were performed in 7 to 10 days old Wistar rat pups, the equivalent to 35-to-38 week-old human newborn (Semple et al., 2013). The experiments were designed to use the minimal number of animals to achieve statistical significance.

In order to avoid rejection, dam was conditioned previously to the surgical procedure for the HI experiment. The dam and its litter were kept in different cages for ten minutes and then reunited for another ten. That was repeated twice, and in every separate time pups were sprayed with chlorhexidine, the antiseptic used after the procedure, so the dam may get used to the odour. Importantly, during the second separation the dam was kept in another room. Pups from each litter were randomly assigned to the surgical protocol or control.

1.1 Hypoxic-Ischemic brain damage induction

As explained before, the aim of this animal model is to reproduce the diffuse brain damage caused by NHIE. HI brain damage animal model was based on Rice-Vannucci protocol (Vannucci & Vannucci, 2005) with slight modifications (Pazos *et al*., 2012). On postnatal day (PND) 7 to 10 Wistar rats were anesthetized by sevofluorane (5% induction, 1% maintenance) and their left carotid artery was isolated and electrocoagulated. Then they were returned to their dam for 3 hours to recover. After that, pups were placed into 500mL jars in groups of three and exposed to hypoxia (10% fraction of inspired oxygen) for 112 minutes (HI-operated animals). Jars were kept in a warm water bath at 37ºC in order to avoid hypothermia. For control group (SHAM-operated animals), similar surgical procedure was performed but without carotid electrocoagulation or hypoxia (Fig.11).

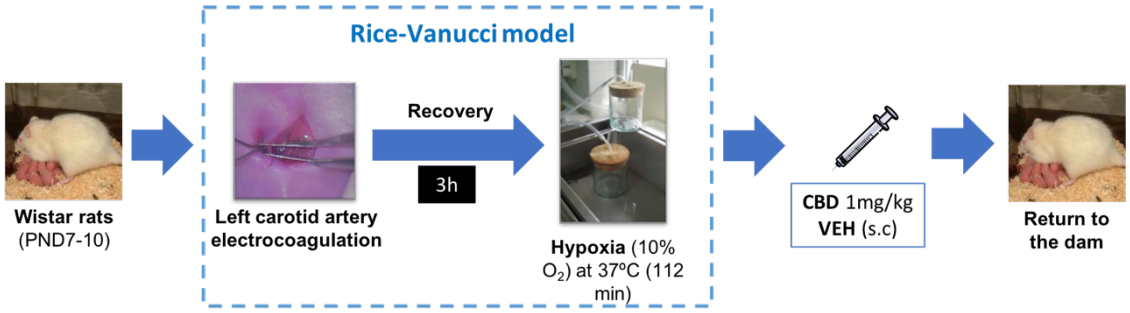


Figure 11. Rice-Vanucci animal model protocol

Ten minutes after the end of hypoxia or equivalent period, SHAM and HI rat pups were randomly treated with a subcutaneous (s.c) injection of vehicle (HV) or CBD 1mg/kg (HC), as previously described by Pazos et al., 2012 (Fig.1). Following drug administration, pups returned to their cage with their dam for 24 hours (24h) or 7 days (7 d). In the case of pups followed for up to 30 days (30 d), weaning was at PND21 and then males and females were grouped separately.

For CB_2 experiment, the specific CB_2 agonist HU-308 and the antagonist/inverse agonist SR144528 (SR2) were chosen. The HU-308 dose was previously optimized by a dose curve from 1 to 10 mg/kg due to the high variability of doses found in bibliography, while SR2 was used at 3 mg/kg (Fujii et al., 2014a; Tao et al., 2015; Maria Teresa Viscomi et al., 2009). Six different experimental group were designed: VEH/VEH (HI+VEH), VEH/CBD (HI+CBD), VEH/HU-308 (HI+HU), SR2/VEH (HI+SR2), SR2/CBD (HI+SR2+CBD), SR2/HU-308 (HI+SR2+HU) and HU-308/CBD (HI+HU+SR2). Following the HI protocol previously described, ten minutes after the end of hypoxia, HU-308, SR2 or vehicle were administrated and ten minutes later, well CBD 1mg/kg or VEH were co-administrated. Finally, pups were returned to their dam for 7 days before sacrifice (Table 2).

CB2 Ligands	Standard treatment
VEH	CBD
SR2	
HU-308	
VEH	VEH
SR2	
HU-308	

Table 2. CB_2 experiment groups

1.2 Temporary Middle Cerebral Artery Occlusion

This model is an adaptation of the well-known stroke model in adult rodents adapted for 7-to-9-day old rats by Derugin et al. (Derugin et al., 1998). Each pup was anesthetized by sevofluorane (5% induction, 1% maintenance), and the left carotid was dissected up the internal and external branches. A small cut was done at the beginning of the internal carotid artery, then an occluder (0.21mm of diameter) was introduced until it stucks at the origin of the left middle cerebral artery (8.5-9mm). Then, the wound was sealed, the pup awakened for a three-hour-long

occlusion period. Then, pups were anesthetized again and the occluder removed. The carotid was quickly sealed with collagen to avoid bleeding. During all the procedure pups were kept at 37-38ºC using a heat blanket. Control group (SHM) undergo the same protocol without left carotid dissection or middle cerebral artery occlusion. Ten minutes after occluder removal animals were randomly assigned to receive CBD (MCAO-CBD) or vehicle (MCAO-VEH) intraperitoneally (i.p.) (Fig. 2). Different doses of CBD, such as 1, 5, 10, 50 or 100 mg/Kg. Finally, pups were returned to their dam and weaned for 7 or 30 days.

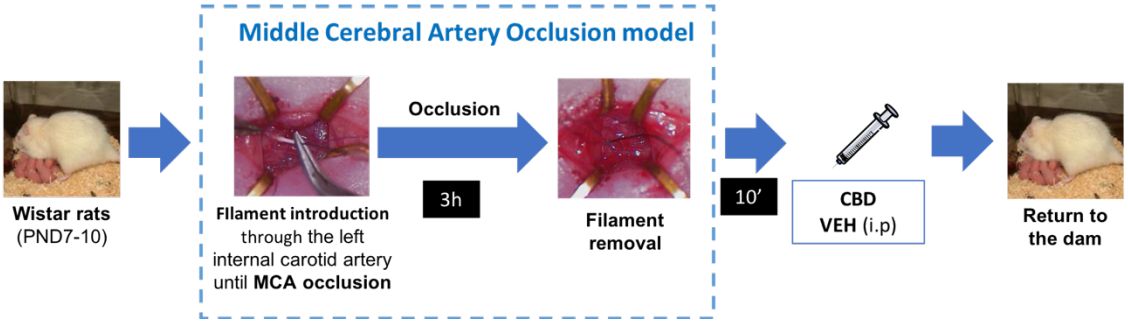


Figure 12. Middle Cerebral Artery Occlusion protocol

1.3 Drug preparation

CBD and VEH were a gift from GW Research Ltd (Cambridge, UK) while HU-308 was a generous donation from Prof. Raphael Mechoulam of Institute for Cannabinoid Research at the Medical Faculty in the Hebrew University (Jerusalem, Israel). Because of cannabinoid hydrophobicity all plastic tubes were pre-treated with Sigmacote (Sigma-Aldrich; San Luis, USA).

For the HI and MCAO models, CBD was prepared as follows: CBD was dissolved in ethanol and then Solutol (BASF SE, Ludwigshafen, Germany) and saline were slowly added at 60ºC to a final proportion of ethanol:solutol:saline 1:1:17. Finally, the stock solution at 5mg/mL was aliquoted and immediately stored at -20ºC.

For PAIS experiments both CBD stock at 3 mg/mL concentration and VEH were supplied by GW Research Ltd (Cambridge, UK). The injectable solution was prepared the day of the experiment by diluting the stock at 3mg/mL with saline to the desired dose.

HU-308 and SR2 were directly dissolved in ethanol, gassed with nitrogen to avoid oxidation and rapidly stored at -20ºC as a stock solution containing HU-308 40mg/mL or SR2 47.62mg/mL. The day of the experiment HU-308 or SR2 were further diluted in Solutol or Tween-80 (Sigma-Aldrich; San Luis, USA) and saline to a final proportion of 1:1:17.

In all cases the injectable solution was prepared in order to administrate 0.1mL per animal at the desired concentration.

2. NEUROBEHAVIORAL TESTS

To evaluate functional impairment after HI and MCAO models, different tests were performed seven and thirty days after the insult. Due to both motor and cognitive skills are not fully developed at PND14-PND17, only motor reflexes and basic motor coordination were assessed at this age. One the other hand, at least two motor and one cognitive test were performed in each model thirty days after the insult, in order to evaluate neurobehavioral impairment. The functional tests were analyzed by two different examiners blinded to the experimental group.

2.1 Neonatal rat motor tests.

Seven days after the brain damage several motor test were performed to analyze motor reflexes and motor coordination (Bouet et al., 2010; Heyser, 2004) (Fig. 13):

- Negative geotaxis: the negative geotaxis test was developed to asses motor coordination in young rat. Each pup was placed facing down a 45º slope and the time required to turn up the slope was measured. Animals with greater motor affectation will used more time to complete the task (Ten et al., 2003).
- Front-limb suspension test: this test was applied to measure the forelimb strength of pups. Briefly, pups were suspended hanging from a wire with both forelimbs. The time to fall is recorded, with smaller time in animals with poorer outcome (Fan et al., 2005).
- Grip reflex: the goal of this test is analyzing the reflex lost in contralateral limb as consequence of unilateral brain damage. The rat was held by the experimenter and a thin rod is used to measure the grasp reflex in both fore- and hind-limbs. Being 2 = digit flexion; 1 = partial or delayed (more than 2 seconds) digit flexion; 0 = digit extension (Bouet et al., 2010).
- Grasp reflex: this test measures the reflex of getting a support surface when the pup is held in the air by the examiner. With the rat held by the experimenter by the neck, a rod is approximated to both hind-limbs and the reflex of carrying its own weight is measured. Being 2 = full weight; 1 = partial; 0 = no movement.

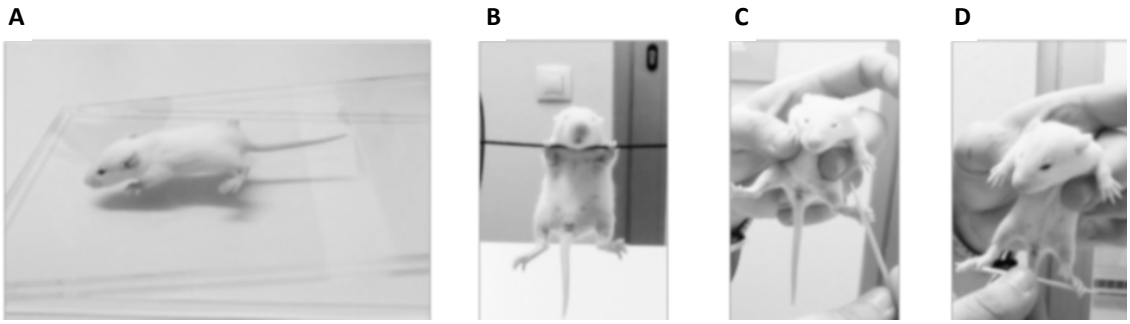


Figure 13. Motor Reflexes and Motor Coordination tests seven days after damage. A) Negative geotaxis; **B)** Fore-limb suspension test; **C)** Grip reflex; **D)** Grasp reflex.

2.2 Adult rat motor and cognitive tests.

One month after the brain damage different cognitive and sensorimotor tests were used (Bouet et al., 2010; Pazos et al., 2012; Russel et al., 2011; Schaar et al., 2010) (Fig.14)**:**

- Beam test: to measure motor and coordination deficits (Patel et al., 2015; Russel et al., 2011; Schaar et al., 2010; Woodworth et al., 2011). In this test the rat was encouraged to cross a beam 1 m-long with a flat surface 1 cm wide held at 50 cm height. At the start point of the beam, a strong white lamp was used to incite the rat movement while a dark chamber was located at the end. A training day was necessary to avoid rat turns or stops, in which rat first crossed by a close platform followed by crossing the beam (Schaar et al., 2010). The rat was let in dark chamber with palatable food for 10 seconds each time. The time required to cross the beam was measured.
- Cylinder Rearing test (CRT): to asses lateral bias of motor deficits (Kim et al., 2017; Woodworth et al., 2011). The animal is placed in a transparent cylinder (20 cm in diameter and 30 cm in height) for five minutes and the number of each forepaw placing in the surface of the cylinder is counted. The possible bias to the left -ipsilateral- forepaw use was calculated as [(left - right)/(left + right + both)]*100. At least four wall contacts per rat were necessary for trial analysis.
- Adhesive Removal Test: to analyse motor coordination and fine sensorimotor deficits (Schaar et al., 2010; Woodworth et al., 2011). Rats were previously habituated to the cylinder (20 cm in diameter and 30 cm in height). Equal adhesive tapes were placed in each forepaw with the same force and the time required to detect -showed by a forepaw's contact- is measured (Bouet et al., 2010).

- Novel Object Recognition test (NOR): to asses non-spatial working memory (Patel et al., 2015; Pazos et al., 2012). Habituation to the cage (40 x 40 x 30 cm) was done the day before the experiment. On the texting day, the animal was previously placed in the cage with two identical objects for five minutes. Rats rested for an hour and then returned to the cage where one object had been replaced by a new one for the trial. The trial lasted five minutes and was recorded for forthcoming quantification of the exploring time of each object. The discrimination index was calculated as: $(Time_{new\ object} - Time_{familiar\ object})/(Time_{new\ object} + Time_{familiar\ object})$.

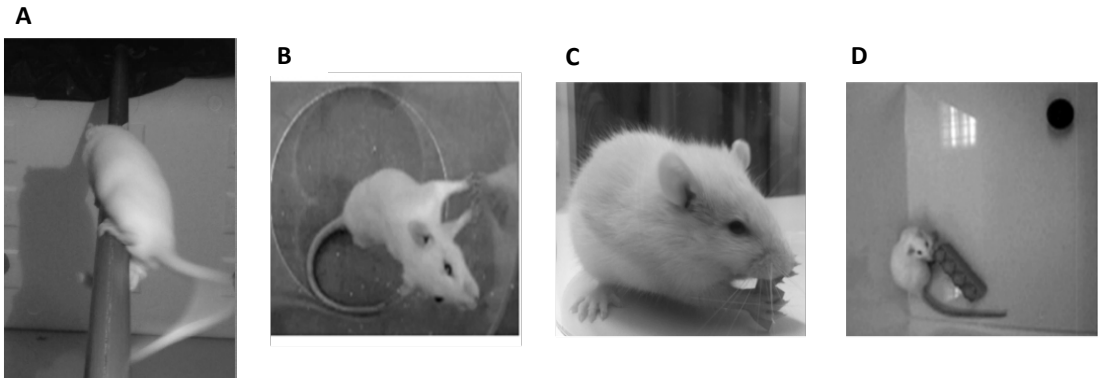


Figure 14. Neurobehavioral tests performed thirty days after damage. A) Beam test; **B)** Cylinder Rearing test; **C)** Adhesive Removal test; **D)** Novel Object Recognition test.

3. SAMPLING

After the functional tests, rats were sacrificed under deep anesthesia (diazepam:ketamin [i.p.]) and transcardially perfused with 0.1M phosphate-buffered saline, pH 7.4 (PBS) followed by 4% paraformaldehyde to perform MRI followed by immunohistochemical studies. For the electron microscopy studies, rats were perfused with PBS, followed by the fixative solution made up of 4% formaldehyde, 0.2% picric acid, and 0.1% glutaraldehyde in PBS. Brains remained in the fixative solution for 1 week at 4°C and then stored at 4°C in a 1:10 diluted fixative solution until use.

For Western-Blot experiments and Magnetic Resonance Spectroscopy (H^+-MRS), 7 days post-HI rats were sacrificed by carbon dioxide followed by a rapid decapitation. Brains were dissected, snap-frozen in isopentane and stored at -80ºC.

4. MAGNETIC RESONANCE IMAGING

MRI is a well-known system to quantify brain damage and oedema (Larpthaveesarp and Gonzalez, 2017; Pazos et al., 2012). Brain MRI scans were performed at MRI Unit of the Instituto

de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM, Madrid, Spain) on a BIOSPEC 70/16 (Bruker-Medical, Ettlingen, Germany) or at the CAI de Resonancia Magnética Nuclear y Espín Electrónico (Instituto Multidisciplinar, Universidad Complutense de Madrid) on a BIOSPEC 47/40 (Bruker-Medical, Ettlingen, Germany). MTI devices operates at 4.7 T, equipped with an actively shielded gradient insert with an 11.2 cm bore, a maximal gradient strength of 200 mT/m, an 80 µs rise time, and a home-made 4 cm surface coil. T2WI was acquired with a multislice rapid acquisition (TR = 3.4 s, RARE factor = 8, interecho interval = 30 ms, TEeff = 120 ms; matrix size = 256 x 256 (pixel dimensions 117 x 117 µm), field of view (FOV) = 3 cm2). The slice package consisted of 26 consecutive 0.5-mm-thick slices in the axial plan with an interslice gap of 0.1 mm to image the entire brain. To perform the MRI scan, brains were placed in Fluorinert FC-40 (3M, Minnesota, USA) and then re-placed in paraformaldehyde .

Both the entire brain volume loss and the percentage of high intensity signal area were analysed using the ImageJ 1.43u software (National Institute of Health [NIH], Bethesda, USA) (Pazos et al., 2012).

5. MAGNETIC RESONANCE SPECTROSCOPY

Frozen samples from ipsilateral cortex were used for H^+-MRS analysis. H^+-MRS was performed on a Bruker AMX500 spectrometer 11.7 T at the MRI Unit of the Instituto de Investigaciones Biomédicas "Alberto Sols" (CSIC-UAM, Madrid, Spain). The device operates at 4ºC on frozen samples (3 mg) placed within a 50-µl zirconium oxide rotor with a cylindrical insert and spun at 4000 Hz. Standard solvent-suppressed spectra were acquired as 16 k data points and averaged over 256 acquisitions. The total acquisition time was~14 min using a sequence based on the first increment of the NOESY pulse sequence to affect suppression of the water resonance and limit the effect of Bo and B1 inhomogeneities in the spectra (relaxation delay-90º-t1-90º-tm-90º-acquire free induction decay), in which a secondary radio frequency irradiation field was applied at the water resonance frequency during the relaxation delay of 2s and during the mixing period (tm = 150 ms), with t1 fixed at 3 µs. A spectral width of 8333.33 Hz was used. All spectra were processed using TOPSPIN software, version 1.3 (Bruker Rheinstetten, Germany). Prior to Fourier transformation, the free induction decays were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz. Spectra were phased, baseline-corrected and referenced to the sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate singlet at δ 0ppm.

SpinWorks 3.1.7.0 software were used to analyze the data by curve fitting. Several ratios were calculated: lactate/N-acetylaspartate (Lac/NAA), glutamate/N-acetylaspartate (Glu/NAA),

gluthatione/creatine (GSH/Cr) and myoinositol/creatine (mI/Cr) ratios to analyze changes in phosphorylation potential (Li et al., 2010; Penrice et al., 1997b), excitotoxicity (Groehandal *et al*., 2010), oxidative stress (Satoh and Yoshioka, 2006) and astrocyte function (Harris et al., 2015), respectively.

6. HISTOCHEMICAL ANALYSES

After MRI analysis, brains were processed and embedded in paraffin. Coronal sections of 4µm of width were obtained using a microtome and the chosen area of study was from plate 14 to 18 of Paxinos (Paxinos and Watson, 1996). For HI experiment immunohistochemical studies were performed in the ipsilateral parietal cortex, corpus callosum (external capsule), as examples of Grey and White Matter areas damaged by HI, and the subventricular zone (SVZ) since is one of the main proliferative niches of the brain and it is responsible for the OPC generation that lately migrate and differentiate into white matter areas (Mao et al., 2013). For MCAO experiments, the ipsilateral parieto-occipital cortex adjacent to the infarct area was analysed (Fernández-López et al., 2013a).

6.1 Nissl staining

For Nissl staining, a toluidine blue solution was utilized as a basic dye which binds to Nissl bodies in neurons and nucleic acids. Widely used to stain neuronal bodies, it allows to asses neuronal damage after HI by identifying pyknotic/necrotic neurons. Firstly, tissues were deparaffinized and rehydrated. Then, tissues were dept in toluidine blue (0,5% in distilled water) for 30 seconds. After that, tissues were destained with water and increasing alcoholic solutions to finish the dehydration in xylene. Finally, it slides were mounted with non-aqueous mounting medium DPX new (Merck Millipore; Billerica, USA) and let dry.

Parietal cortex was examined using a light microscope (200X) and neuronal damage were assessed by a damage score from 0 to 5. Being 0, less than 2% of cell death; 1, few cells damaged (<25%); 2, several neurons damaged (25-40%); 3, moderate damage (40-65%); 4, severe damage (> 65%), and 5, total cell death or absent tissue. Damaged neurons were identified when no distinction could be made between cytoplasm and nucleus or as shrunken neurons, with a high stained center surrounded by tissue loss. By contrast, apparently healthy neurons are characterized by a differentiated cytoplasm, soft stained, and nucleus, darker and with distinct nucleolus. The analysis was performed by two researchers blinded to the experimental group (Alvarez et al., 2008; Pazos et al., 2012).

6.2 Immunohistochemical analyses

Tissue sections were deparaffinized and rehydrated, and then heat-induced antigen retrieval was made by using citrate solution (pH6, 10mM) or Tris-EDTA (pH9, TRIS 10mM-EDTA 1mM) with 0.05% of Tween-20 buffer at high temperature and pressure. Tissues were washed with phospahate buffer saline (PBS) followed by an ammonium chloride (10mM) incubation for ten minutes at room temperature. After several PBS washes, brain sections were incubated overnight at room temperature with the primary antibody in a humidified chamber. The different primary antibodies used are shown in Table 3. After extensively washes, tissues were incubated with the corresponding secondary antibody (1:200, α-rabbit, α-mouse or α-goat Alexa-Fluor 488 or 546 (Molecular Probes, Oregon; USA) for 2 hours at 37ºC in a humidified chamber. TO-PRO (1:500; Life Technologies Spain) was used for nucleus staining. Slides were finally mounted with Glicerol:PBS aqueous mounting medium.

Target	Antibody	Company	Dilution
Astrocytes	GFAP	Sigma-Aldrich; San Luis, USA	1:1000
Microglia	Iba-1	Wako; Osaka, Japan	1:400
Neurons	NeuN	Merck Millipore; Billerica, USA	1:300
Neuron Progenitor	NeuroD1	Abcam; Cambridge, UK	1:200
Mature oligodendrocyte	GST-π	Abcam; Cambridge, UK	1:100
OPC	Olig2	R&D Systems; Minneapolis, USA	1:100
PreOL	SOX10	Santa Cruz Biotechnology; Dallas, USA	1:100
Myelin Basic Protein	MBP	Sigma-Aldrich; San Luis, USA	1:800
Synthetic nucleoside 5-bromo-2'-deoxyuridine	BrdU	Novus Biologicals; Littleton, USA	1:200
Proliferating cells	Ki67	Master Diagnostica; Granada, Spain	1:400

Table 3. Primary antibodies for immunohistochemical studies.

To analyze proliferation after HI, the synthetic nucleoside 5-bromo-2'-deoxyuridine (BrdU) was used (Sigma-Aldrich; San Luis, USA). This nucleoside is an analog of thymidine and, as a consequence, it will be incorporated into the new synthesized DNA strains, thus marking the new cells produced during the treatment period. Rat pups were injected with BrdU (50mg/kg, dissolved in saline) in days 4, 5 and 6 after HI damage, as has been previously described (Fernández-López et al., 2013b, 2010), and the animal euthanized at day 7 after damage.

Tissues were photographed using a TCS SP5 confocal microscope (Leica Microsystems, Germany). Analyses were performed by an examiner blinded to the experimental group using the ImageJ 1.43u software (NIH, Bethesda, USA) to calculate the cell density and signal intensity. In addition, MBP intensity of signal was determined with the LEICA LASF Software (Leica Microsystems, Germany), and expressed as ratio of Ipsilateral MBP vs total (ipsi+contralateral) MBP immunostaining due to interassay and animal autofluorescence variabilities (Liu et al., 2002).

6.3 TUNEL immunohistochemical staining

DeadEnd™ Fluorometric TUNEL System (Promega; Madison,WI, USA) was used for TUNEL staining. This technique specifically labels the free 3-OH termini of DNA strains in situ after DNA fragmentation caused by endonucleases during apoptosis. Nevertheless, although DNA fragmentation precedes cell death, if it is by apoptosis or another kind of cell death, cannot be differentiated by TUNEL technique alone. Briefly, modified nucleotides, fluorescein-12-dUTP[(a)] are incorporated to DNA 3-OH end by terminal deoxynucleotidyl transferase (TdT) until it forms a fluorescent oligomer which can be directly observed by fluorescence microscopy. Firstly, tissue sections were deparaffinized, washed for 10 minutes in sodium chloride and finally washed with PBS. Then proteinase K (20μg/mL in PBS, 100μL/tissue) was applied for 8-10 minutes at room temperature to permeabilize tissue, followed by three PBS washes. After that, Equilibration Buffer was applied for 5 to 10 minutes followed by the incubation with TdT enzyme and the modified nucleotides, fluorescein-12-dUTPs, for 1 hour at 37ºC in a humified chamber. Then the reaction was stopped by the stop buffer, and the tissues washed several times with PBS and deionized water. After washes to remove unincorporated modified nucleotides, samples were incubated with TO-PRO (1:500; Life Technologies Spain) for 10 minutes and then, wash again. Finally, slides were mounted with Glicerol:PBS aqueous mounting medium.

6.4 Electron Microscopy studies

Electron microscopy studies were performed at Prof. Pedro Grandes' laboratory (School of Medicine and Nursery, Universidad del País Vasco; Bilbao, Spain). Animals were sacrificed and perfused, and the brain obtained as previously explained in sampling. Brain coronal sections were cut at 50 µm in a vibratome and collected in PBS at room temperature. Sections were osmicated (1% osmium tetroxide in PBS pH 7.4) for 60 minutes at 0°C, dehydrated in graded alcohols to propylene oxide, and plastic-embedded in Epon resin 812. Ultrathin sections of 60nm were collected on mesh nickel grids, stained with 2.5% lead citrate for 20 minutes, and examined in a Philips EM208S electron microscope. Tissue preparations were photographed by using a digital camera coupled to the electron microscope. Three animals were analyzed per treatment and the ultramicrographs were analyzed by the ImageJ 1.43u software (NIH, Bethesda, USA).

To calculate the number of axons electron micrographs were taken at x5,600 magnification and 10 photos were made per animal of the two analyzed areas, external capsule and cortex. The analyzed area per photo was 308 μm^2. For myelin sheath thickness analysis and g-ratio calculation, micrographs were taken at x11,000 magnification. Firstly, the fiber perimeter of the axon was marked. Later, the ratio distance from the center of the axon to the fiber and myelin sheath perimeter was measured in four different point of the axon, and its average calculated. Ten axons per animal were analyzed. The number of axons analyzed and the magnification of the micrographs were the same for external capsule and cortex.

The g-ratio is a parameter used as a structural index of axonal myelination. The g-ratio was calculated by dividing the measured inner (axon) perimeter by the measured outer (myelin) perimeter of all axons in each micrograph. Since the form of the axons is sometimes very variable, the use of the perimeter instead of the diameter is more reliable (Ford et al., 2015) (Ford et al., 2015). For the g-ratio the axon perimeter and the fiber perimeter of all the axons were analyzed in each micrograph. An average of 150 axons per treatment in the external capsule and an average of 100 axons in the cortex were analyzed. The axons used to analyze the g-ratio were also used to analyze the axon perimeter and area.

7. WESTERN-BLOT

For protein extraction, frozen tissues were homogenized in tissue protein extraction reagent (T-PER, 1 g of tissue/10 mL; Pierce Biotechnology, Rockford, USA) and centrifuged at 10,000g for 5

min at 4ºC. Pierce BCA Protein Assay Kit (Pierce Biotechnology; Rockford, USA) was used to quantify the protein concentration.

20μg of denatured protein of each sample were separated on 12% acrylamide/bis-acrylamide gels (TGX Stain-Free Acrylamide Kit 12%, Bio-Rad; Hercules, USA). Stain-Free gels allows the direct quantification of protein loaded per lane, increasing internal control accuracy and saving the use of another protein control quantification such as β-actin. After electrophoresis Proteins were transferred to polyvinylidene fluoride membranes (GE Healthcare; Little Chalfont, UK), and total protein loading was visualized by ultraviolet exposition. Then membranes were blocked for one hour in TBS (TRIS 25mM pH7.4, NaCl 1.37M) -Tween 0.1% containing 5% nonfat dried milk at room temperature. Subsequently, blots were incubated with the primary antibody overnight at 4ºC. The different primary antibodies used for western-blot studies are listed on Table 4. Membranes were finally incubated with the corresponding HRP-labeled secondary antibody (1:5000; GE Healthcare; Little Chalfont, UK) for 1h at room temperature. An enhanced chemiluminescence Kit (GE Healthcare; Little Chalfont, UK) was used for the peroxidase reaction and a Gel-Doc station with Quantity One 4.5.1 (basic) analysis software (Bio-Rad; Hercules, USA) was used for detection. Resultant images of both target protein and loaded protein were analyzed with ImageLab software (version 6.0.0, Bio-Rad; Hercules, USA) to determine the intensity per mm^2 of each band and lane, respectively. Results of BDNF and GDNF were expressed as the ratio of target protein/total protein. For TNFα, GAPDH was used for loading control, so results are expressed as a ratio of TNFα/GAPDH.

Target	Antibody	Company	Dilution
Brain Derived Neurotrophic Factor	BDNF	Santa Cruz Biotechnology; Dallas, USA	1:300
Glial Cell-derived Neurotrophic Factor	GDNF	Santa Cruz Biotechnology; Dallas, USA.	1:100
Tumor Necrosis Factor Alpha	TNFα	BIO-RAD; Hercules, USA	1:200
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Chemicon International; Temecula USA	1:1000

Table 4. List of primary antibodies for western-blot studies.

7.1 Oxyblot

To study oxidative stress OxyBlot protein oxidation detection kit (Millipore, Iberica; Madrid, Spain) was used according to manufacturer's protocol. This commercial kit allows the quantification of protein carbonyl groups, protein modifications produced by reactive species like ROS, and consequently analyzed the oxidative stress levels of the sample. Briefly, a total protein amount of 10μg was denaturalized using sodium dodecyl sulfate 6% and then, carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone by samples incubating with 2,4-dinitrophenylhydrazine for 15 minutes. After finishing the reaction, β-mercaptoethanol was added to protein reduction. Then, samples were loaded in a 10% TGX-Stain Free gels (Bio-Rad; Hercules, USA).

Western-blot protocol followed was similar to the one described before, but with specific primary antibody for 2,4-dinitrophenylhydrazone groups (1:150) and its secondary (1:300). An enhanced chemiluminescence Kit (GE Healthcare; Little Chalfont, UK), was used for the peroxidase reaction and chemiluminescence was detected in Gel-Doc station with Quantity One 4.5.1 (basic) analysis software (Bio-Rad; Hercules, USA). Images were analyzed with ImageLab software (version 6.0.0, Bio-Rad; Hercules, USA). Relative intensity quantification of each lane was normalized by total protein loading, so results were expressed as OxyBlot/Total protein.

8. ELISA

Insulin-like growth factor 1 (IGF-1) concentration was determined from 20μg of protein samples by a commercial ELISA kit (Cloud-Clone Corp., USA), according to the manufacture's protocol. Briefly, 100μL of samples and standard were added to each well of the kit plate and incubated for two hours at 37ºC. After that, the liquid was aspirated, and the wells were incubated again with 100μL of Detection Reagent A for one hour at 37ºC. Later, Detection Reagent was aspirated, and wells were washed three time with the Washing Solution. Then 100μL Detection Reagent B were added per well and the plate for one hour at 37ºC. After five washes, the plate was incubated with the Substrate Solution for 20 minutes at 37ºC and the stopped with the Stop Solution. Rapidly, the plate was read at 450nm, using a Thermo Scientific Multiskan EX (ThermoFisher Scientific; Waltham,USA).

9. STATISTICAL ANALYSES

GraphPad 5.0 software was used for the statistical analyses (GraphPad Software; La Jolla, USA). Kolmogorov-Smirnov normality test was applied before running statistical test. The results are

presented as the mean ± standard error, except for CB_2 experiment which is presented as the median ± interquartilic range. Statistical comparisons were made by ANOVA tests followed by Bonferroni or Newman-Keuls post-hoc test for multiple comparisons. When data were non-parametric Kruskal-Wallis test was used. For goal 4, due to the small n of the group ANOVA test was completed by t-tests between groups with Bonferroni's correction for significance. To analyze the correlation between neurobehavioral studies and myelin staining intensity, Spearman test was used. A p value less than 0.05 was considered as statistically significant.

Results

1. CBD NEUROPROTECTIVE EFFECTS IN A FOCAL MODEL OF PERINATAL ARTERIAL ISCHEMIC STROKE IN NEWBORN RATS.

Since CBD neuroprotective effect has been previously reported in models of diffuse HI brain damage in newborn rodents (Castillo et al., 2010; Pazos et al., 2012) and piglets (Alvarez et al., 2008; Lafuente et al., 2016, 2011; Pazos et al., 2013), our first goal was to determine if CBD was similarly neuroprotective in a focal model of HI.

1.1 Dose optimization

Since to our knowledge no previous studies have used CBD as treatment for PAIS, the first step of this objective was to determine the optimal CBD protective dose. We performed a dose-response curve testing 1, 5, 50 and 100 mg/Kg. Only the dose of 100 mg/Kg produced a decrease of the survival rate, with a 20% of animal death in the first 24 hours after the stroke.

MCAO was associated to weight loss observable one week after the insult (Fig. 15A). Whereas doses from 5 to 100 mg/Kg did not enhance weight loss, 1 mg/Kg slightly reduced it (Fig. 15A). Accordingly to these results, MRI studies showed that none of CBD dosages prevented brain volume loss induced by MCAO seven days after the insult (Fig. 15B), although brain volume loss after CBD 1 and 5 mg/Kg treatment tended to be lower than that of VEH-treated animals.

At this early age, only motor reflexes could be tested. In both negative geotaxis and grip tests MCAO led to motor impairment and, although all doses improved negative geotaxis results, only CBD 1 or 5 mg/Kg reduced motor impairment as assessed in both tests (Fig. 15C and D). From a clinical point of view the recovery of motor impairment is very important. Hence, since 5 mg/kg was the lowest dose that showed an improvement in all reflex tests, it was then selected for further studies.

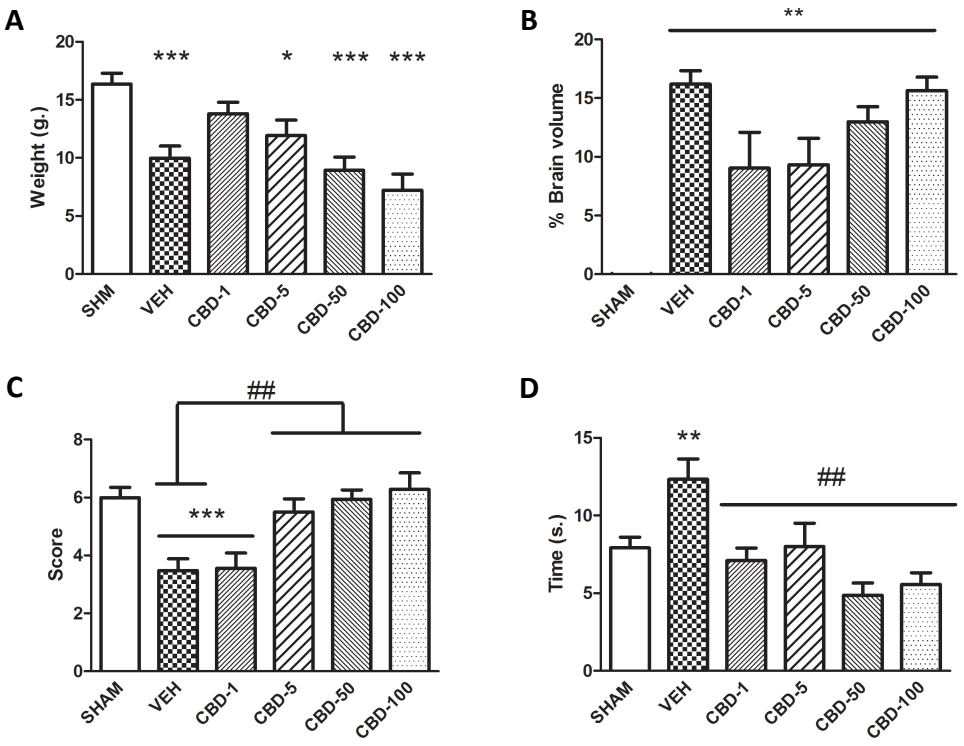


Figure 15. CBD dose determination studies. Different weight and motor reflexes as well as imaging studies were performed 7 days after a perinatal arterial ischemic stroke (PAIS) insult induced in PND7-10 Wistar rats which received vehicle (VEH) or CBD at different doses (CBD-1,5,50,100 mg/kg) and its control littermates (SHM). **A)** Weight gain average; **B)** Percentage of brain volume loss measured by MRI. **C)** Grip reflex teat; **D)** Negative geotaxis test. Bars represent mean±SEM. **p<0.05 vs SHM; ** p<0.01 vs SHM; *** p<0.001 vs SHM; ## p<0.01 vs HV by ANOVA.*

1.2 CBD neuroprotection one week after the insult

To fully characterize motor reflexes, rope test was added to the assessment. MCAO induced a reduction of paw strength which was not averted by CBD administration (Fig. 16).

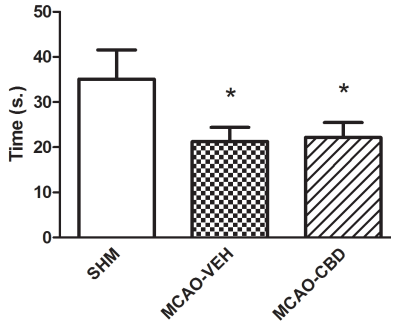


Figure 16. Rope test performance. Duration of the rat holding to the rope, performed seven days after PAIS insult and its treatment with vehicle (MCAO-VEH) or 5mg/kg (MCAO-CBD). Bars represent mean±SEM. ** p<0.05 vs SHM by ANOVA.*

In MRI studies no significant differences were observed in brain volume loss between MCAO-VEH and MCAO-CBD (Fig. 17); however, CBD-treated animals showed a smaller hyperintense area as compared to MCAO-VEH (Fig. 17). In agreement, CBD administration was also able to

prevent MCAO-induced increase of brain lactate and glutamate concentration detected in MCAO-VEH group, keeping those levels similar to SHM (Fig. 18B and C)

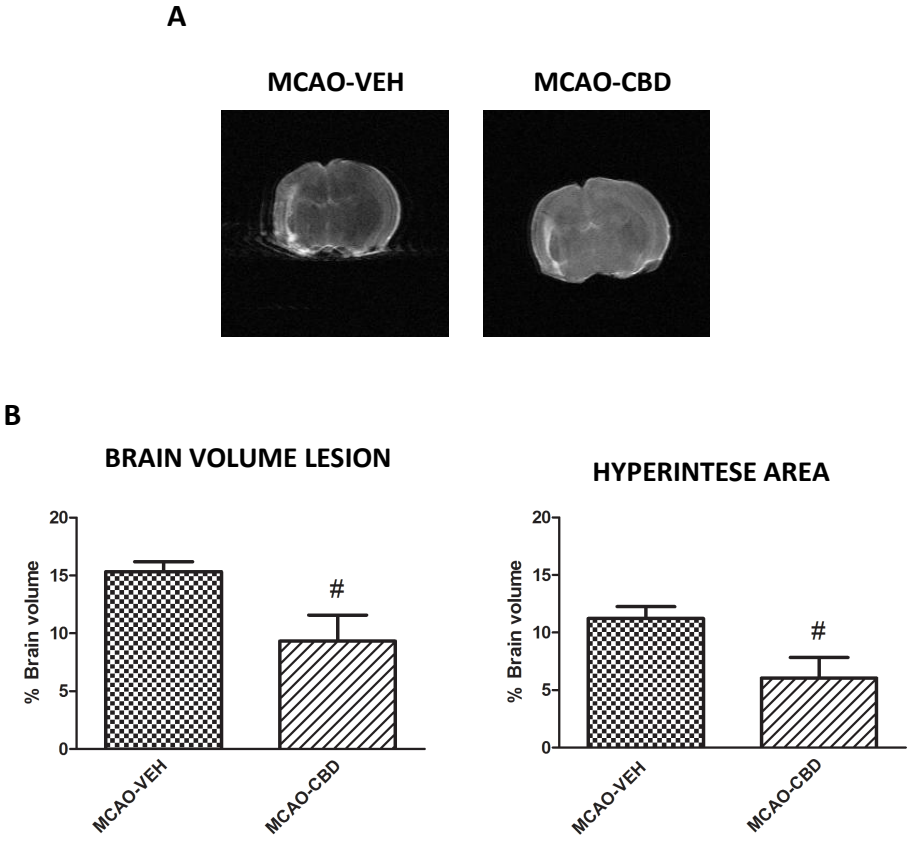


Figure 17. Damage characterization by MRI one week after MCAO. MRI studies performed one week after PAIS insult . **A)** Representative MRI images of rat pups submitted to a MCAO and treated with vehicle (MCAO-VEH) or CBD 5mg/Kg (MCAO-CBD); **B)** Graphical representation of brain volume lesion (left) and hyperintense area (right) quantification. Bars represent mean±SEM. *# p<0.05 vs HV by ANOVA*

Different histological analyses of brain tissue were conducted to asses cell death. Using Nissl staining, it was observed that MCAO rats displayed a higher neuropathological score, independently of the treatment (Fig. 19A). However, when cell death was studied by $TUNEL^+$ cells quantification the increased count observed in MCAO-VEH was remarkably reduced in MCAO-CBD group (Fig. 19B). In agreement, MCAO led to a significant decrease of $NeuN^+$ cells as observed in the perilesional cortex of MCAO-VEH animals, an effect that was prevented by CBD administration (Fig. 19C).

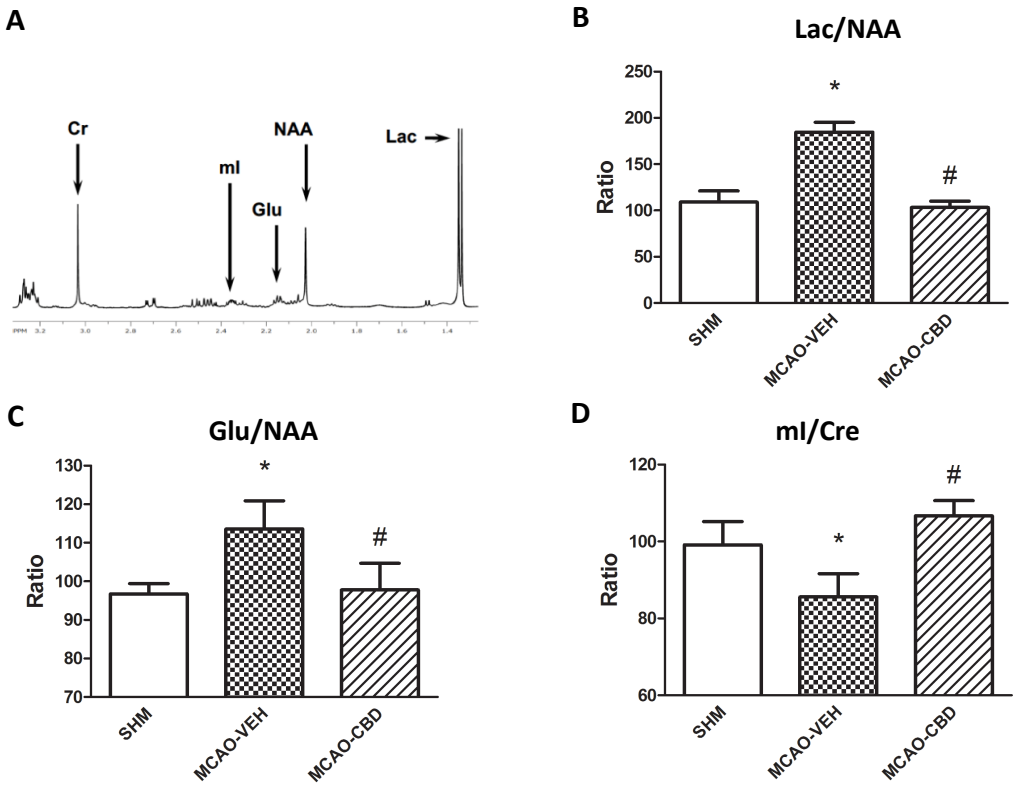


Figure 18. H⁺-RMS studies performed in cerebral cortex. H⁺-RMS analysis carried out on cerebral cortex one week after rat pups were submitted to MCAO. **A)** Representative H⁺-RMS spectre with the analyzed metabolites; **B)** Lactate (Lac/NAA); **C)** Glutamate (Glu/NAA); **D)** myoinositol (mI/Cre). Bars represent mean±SEM. ** p<0.05 vs SHM; # p<0.05 vs HV by ANOVA.*

The study of glial response showed a remarkable increase of GFAP⁺ cells in both MCAO-VEH and MCAO-CBD as compared to SHM (Fig. 20A). MCAO also induced the loss of astrocytic function, as observed by myoinositol reduction, an effect not observed in MCAO-CBD group (Fig. 18D). Besides, microgliosis was also augmented after stroke as shown by the increase of the number of Iba-1⁺ cells (Fig. 20B). MCAO-induced glial density population increase was associated with a more activated morphology phenotype so that in MCAO-VEH those cells showed bigger soma and shorter processes (Fig. 20C and D). By contrast, the administration of CBD reduced both MCAO-induced increase in number (Fig. 20B) and morphological activation (Fig. 20C and D) of Iba-1⁺ cells.

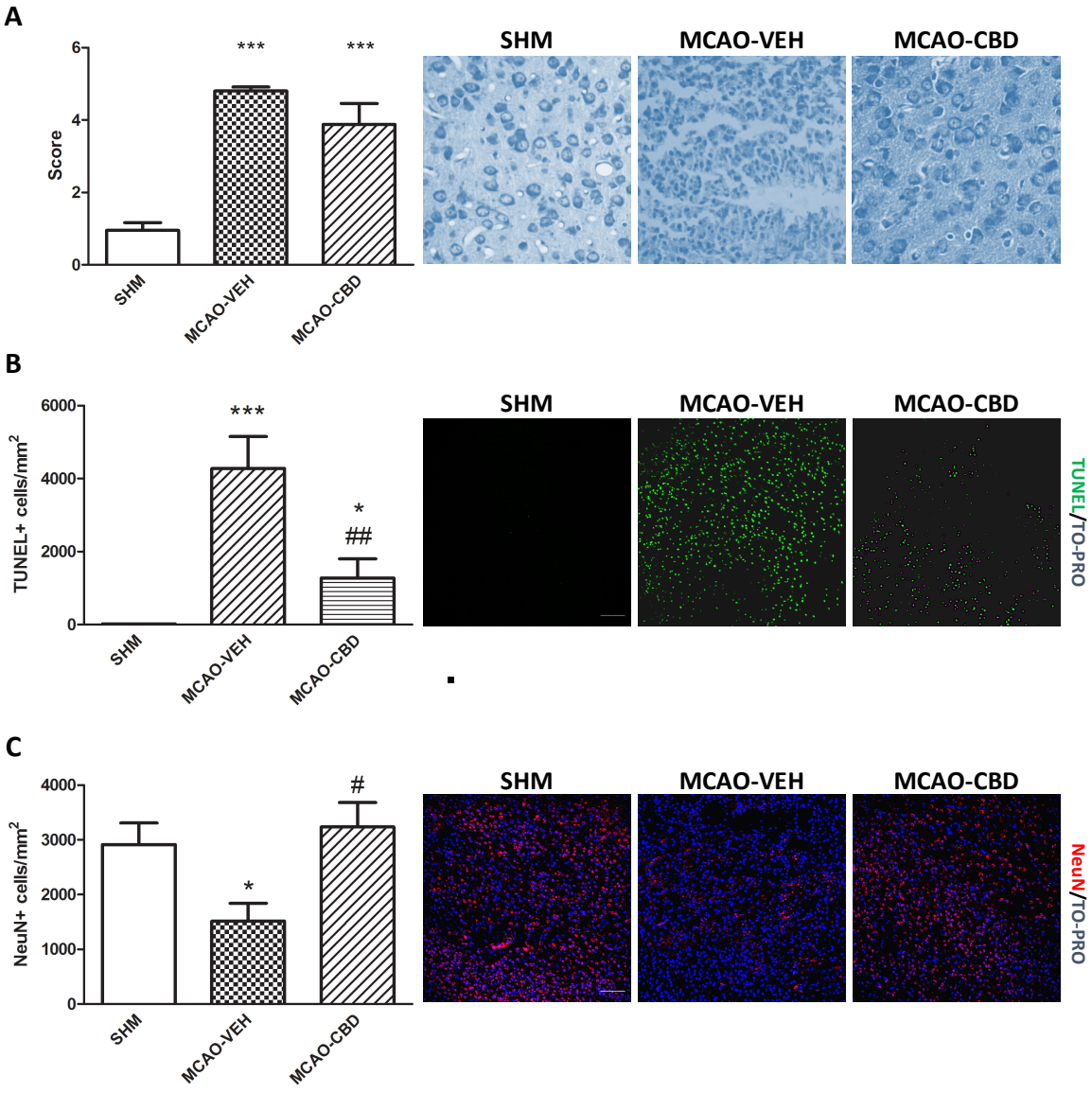


Figure 19. Study of cell death in perilesional cerebral cortex. Representative images *(right)* and graphical representation *(left)* of Nissl and immunohistochemical staining of cell death (TUNEL) and mature neurons (NeuN) performed in the perilesional cortex 7 days after PAIS insult. **A)** Semi-quantitative scoring of cell death using a Nissl staining and **B)**TUNEL positive cell quantification. **C)** NeuN-positive cell quantification. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; *** p<0.001 vs SHM; # p<0.05 vs HV; ## p<0.01 by ANOVA.*

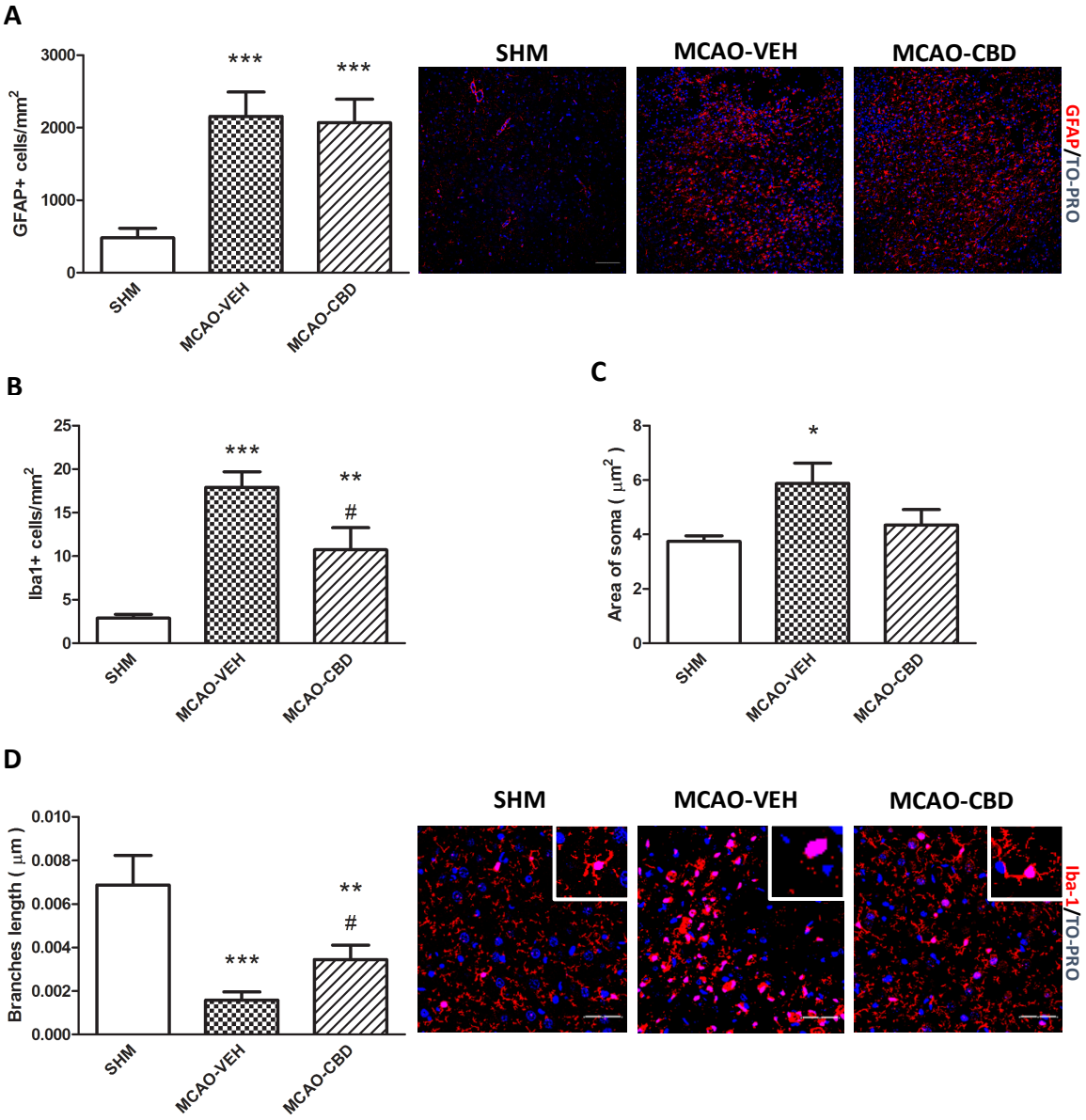


Figure 20. Immunohistochemical gliosis characterization in perilesional cerebral cortex. Immunohistochemical studies performed in the perilesional cortex 7 days after PAIS insult. **A)** GFAP-positive cells quantification *(left)* and representative microphotographs *(right)*; **B, C, D)** Iba-1 staining for microglia cells where Iba1-positive cells were quantified **(B)** and the morphology of the cells analyzed by Iba1-positive cells soma quantification **(C)** and its processes length **(D)**. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; ** p<0.01 vs SHM; *** p<0.001 vs SHM; # p<0.05 vs HV by ANOVA*

1.3 CBD neuroprotection one month after damage

In line with motor reflexes impairment observed one week after the damage, MCAO-VEH animals had poorer performance in beam test, with more time needed to cross the beam (Fig. 21A). CBD administration prevented this motor coordination impairment (Fig. 21A). Moreover, MCAO-VEH animals showed marked hemiparesis and sensitivity loss, as studied by the CRT and

the adhesive removal test, respectively. MCAO-CBD animals scored similarly to SHM in those tests (Fig. 21B and C). No differences were observed in memory tests (NOR) (Fig. 21D).

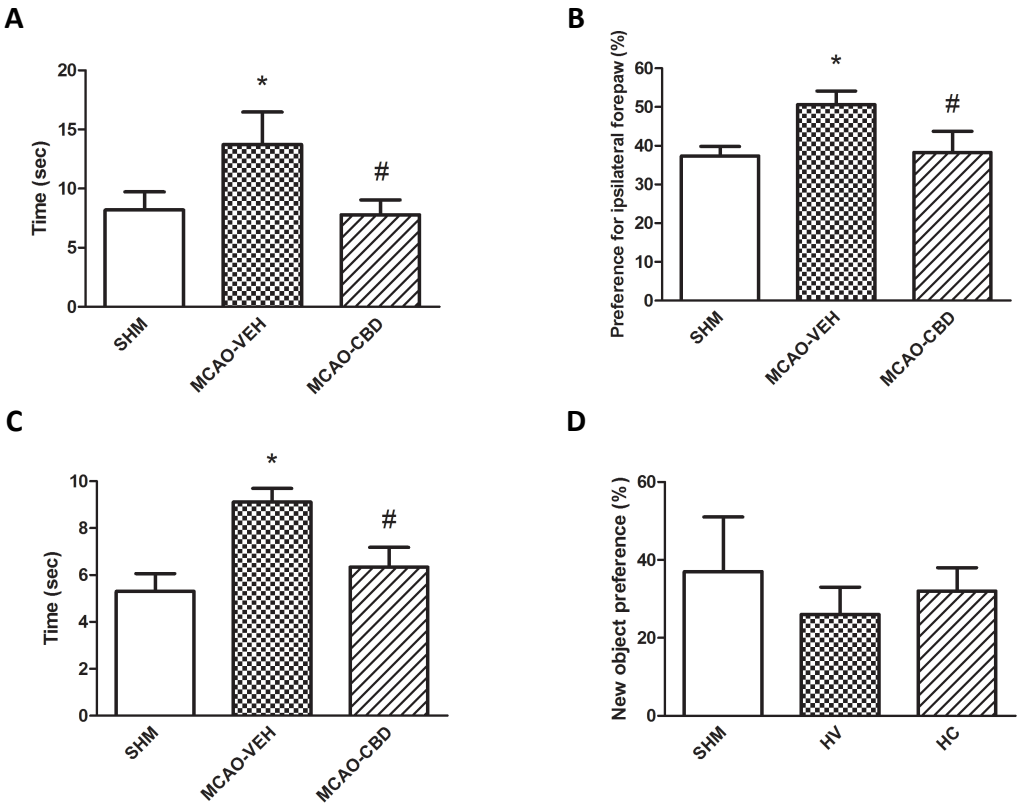


Figure 21. Neurobehavioral tests. Neurobehavioral tests conducted in Wistar rats thirty days after PAIS and being treated with vehicle (MCAO-VEH) or CBD (MCAO-CBD). Its littermates were used as control animals (SHM). **A)** Beam test; **B)** CRT test; **C)** Adhesive test; **D)** NOR test. Bars represent mean±SEM.
** p<0.05 vs SHM; # p<0.05 vs HV by ANOVA.*

In agreement with the aforementioned MRI results one week after brain injury, CBD treatment did not modify the volume of brain loss one month later (Fig. 22A), whereas it significantly reduced the hyperintense area (Fig. 22B). What is more, at this age hyperintense area correlated with a strong astrogliosis which was decreased in MCAO-CBD as compared to MCAO-VEH (Fig. 22C). Noteworthy, one month after the insult a strong microglial response was still observed in MCAO-VEH (Fig. 23); CBD treatment partially prevented the increase in microglia population density (Fig. 23A) but did not modify the morphological alteration (Fig. 23B and C).

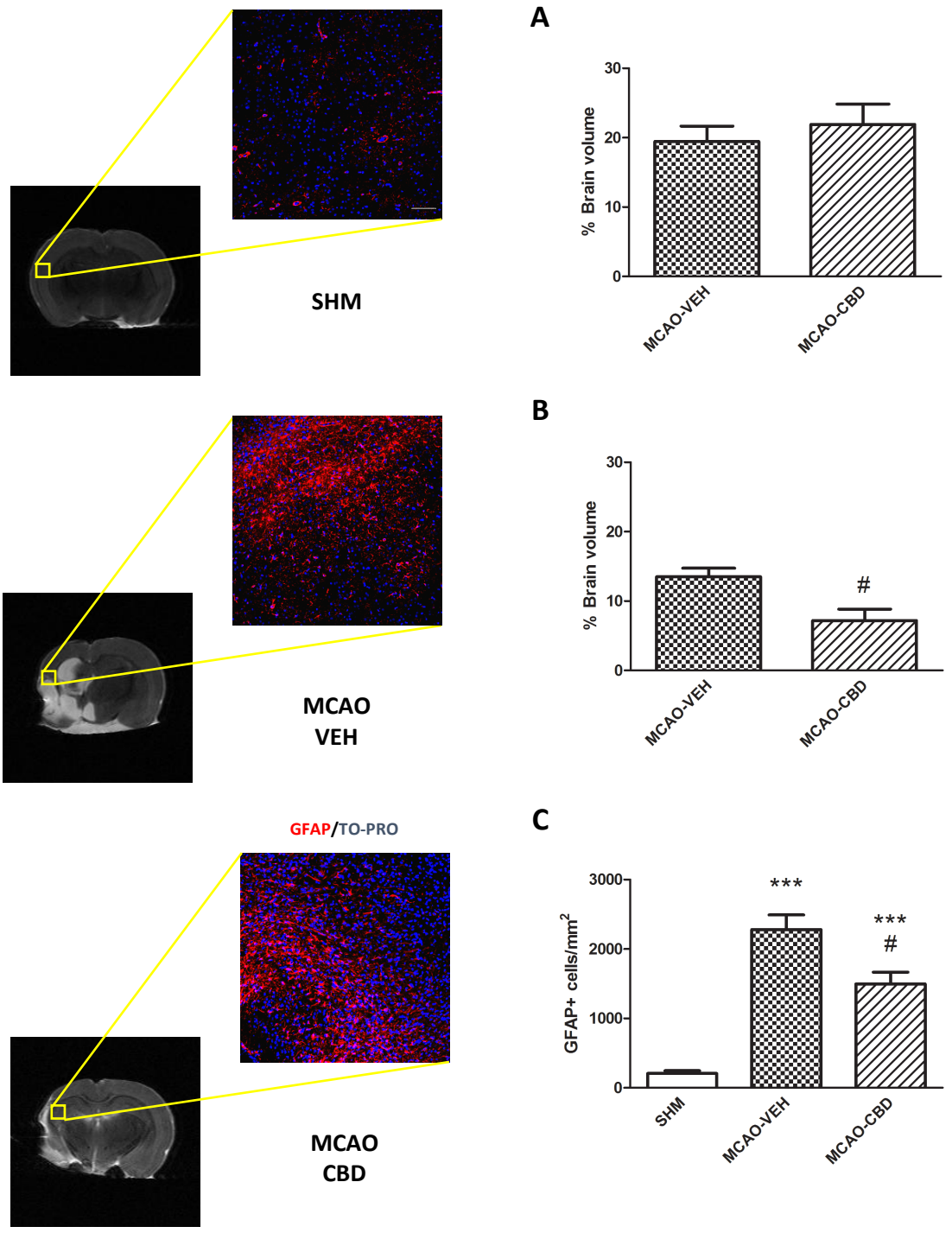


Figure 22. Damage characterization one month after MCAO. MRI and gliosis immunohistochemical analysis performed one month after PAIS insult. Representative images of astroglia scar taken in the hyperintense area observed by MRI. **A)** MRI studies quantifying the brain volume lesion; **B)** Hyperintense area quantification by MRI; **C)** GFAP-positive cell quantification. Bars represent mean±SEM. **** p<0.001 vs SHM; # p<0.05 vs HV by ANOVA.*

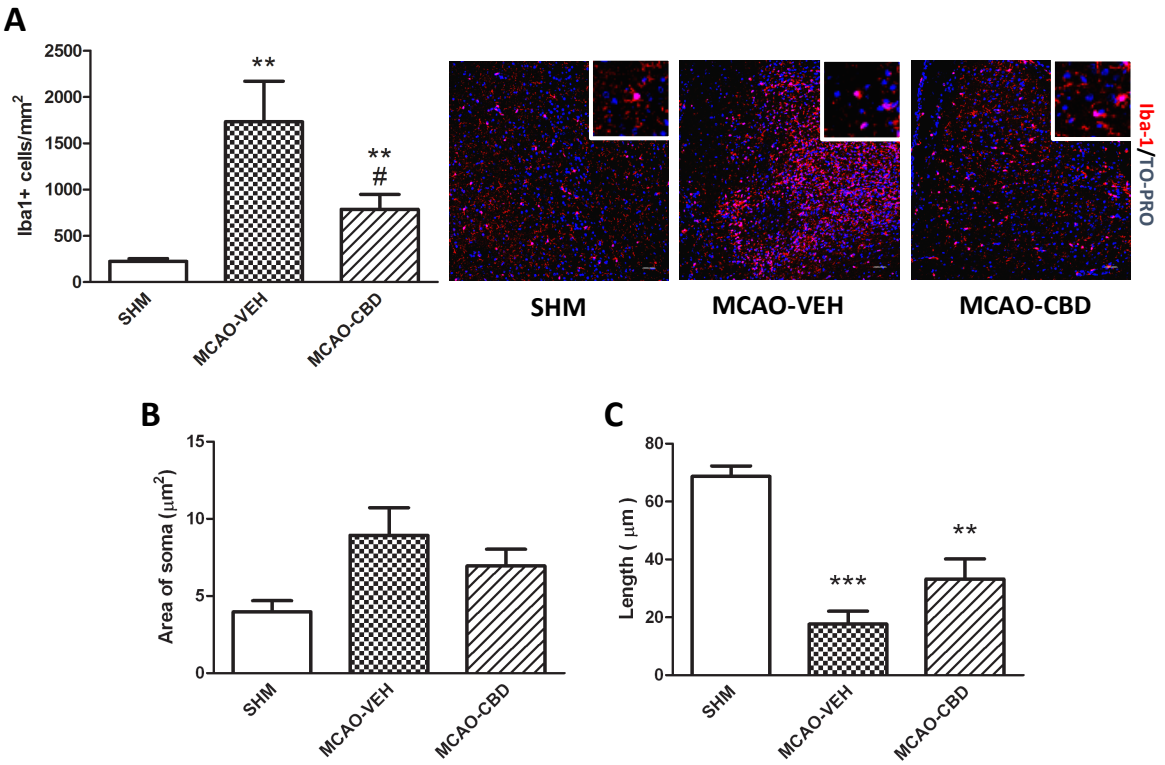


Figure 23. Microgliosis response one month after MCAO. Microgliosis immunohistochemical characterization performed thirty days after PAIS insult. Illustrative microglia population images of Iba-1 positive-cell density and representative microglia cell morphology could be observed . **A)** Iba1-positive cell quantification; **B)** Soma measurement; **C)** Processes length. Bars represent mean±SEM. *** p<0.01 vs SHM; *** p<0.001 vs SHM; # p<0.05 vs HV by ANOVA.*

Finally, MCAO led to both neuron (Fig. 24A) and myelin loss (Fig. 22B) one month after the insult. The effect of MCAO on neurons was reduced by CBD administration (Fig. 24A and B).

1.4 Summary

In conclusion, MCAO induced short- and long- term motor impairment which correlates with brain damage as observed by MRI studies and histological neuron density quantification. Besides, increased cell death labelling and glutamate levels were still observed in perilesional cortex seven days after the insult. Remarkable micro- and astrogliosis was apparent seven and thirty days after PAIS. By contrast, CBD administration prevented the motor impairment induced by MCAO. Although no differences were observed in the volume of brain damage, CBD reduced the hyperintense area as observed in MRI studies, which correlated with the astroglial scar reduction in MCAO-CBD animals observed one month after damage. MCAO-CBD group also

showed lower cell death, excitotoxicity and microgliosis than MCAO-VEH seven days after PAIS, and reduced neuron loss one month after the damage.

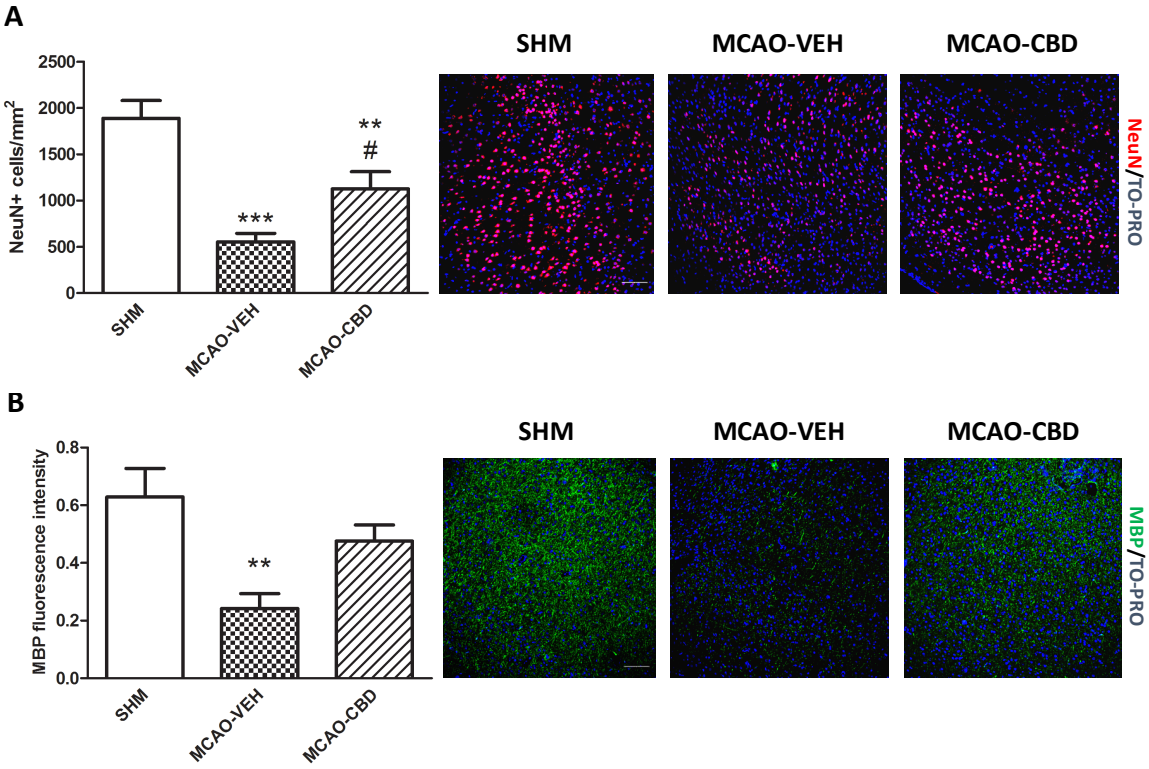


Figure 24. Neuron and myelin study in parieto-occipital cortex. Representative microphotographs and graphical representation of immunohistochemical studies performed one month after PAIS insult in perilesional cortex. **A)** NeuN-positive cells quantification; **B)** MBP intensity signal quantification. Bars represent mean±SEM. Scale bar: 50µm. *** p<0.01 vs SHM; *** p<0.001 vs SHM; # p<0.05 vs HV by ANOVA.*

2. ANALYSIS OF HI-INDUCED LONG-LASTING HYPOMYELINATION IN WHITE AND GREY MATTER AND ITS PREVENTION BY CBD

The long-term hypomyelination detected in the neonatal stroke model in goal 1 has also been observed in models of global HI. Indeed, as it has been described in the introduction, hypomyelination is one of the main deleterious consequences of NHIE in humans. In previous experiments of our group in newborn rats CBD remarkably prevented long-term motor and cognitive impairment as observed in neurobehavioral tests even though only a small reduction of brain volume loss was detected by MRI after HI (Pazos et al., 2012). We hypothesized that this apparently contradictory result might be explained by a possible beneficial effect of CBD on HI-induced hypomyelination. Therefore, hypomyelination studies were performed in the HI model to assess that hypothesis as well as to get more insights into this pathological process in HI model.

To characterize the hypomyelination process after an HI insult and the possible long-term protection offered by CBD administration, two different brain areas were studied one month after HI damage: corpus callosum and parietal cortex.

2.1 Corpus callosum

No differences were found neither in GST-π$^+$ cells density (Fig. 25A) nor in MBP intensity signal (Fig. 25B) among the three groups. However, electronic microscopy analysis revealed that, despite the normal MBP immunohistochemistry, HV animals had fewer axons (Fig. 26A) as well as a thinner myelin sheath (Fig. 26B). Moreover, the surviving axons showed a smaller perimeter in HV than in controls (Fig. 26C). G-ratio was used to avoid myelin thickness analysis bias due to axon perimeter variability, confirming that the g-ratio in HV group was bigger than in SHM group (Fig. 26D).

Administration of CBD after HI blunted that myelin damage, not only preventing the axonopathy (Fig. 26A and C) but also the decrease in myelin thickness (Fig. 26B and D).

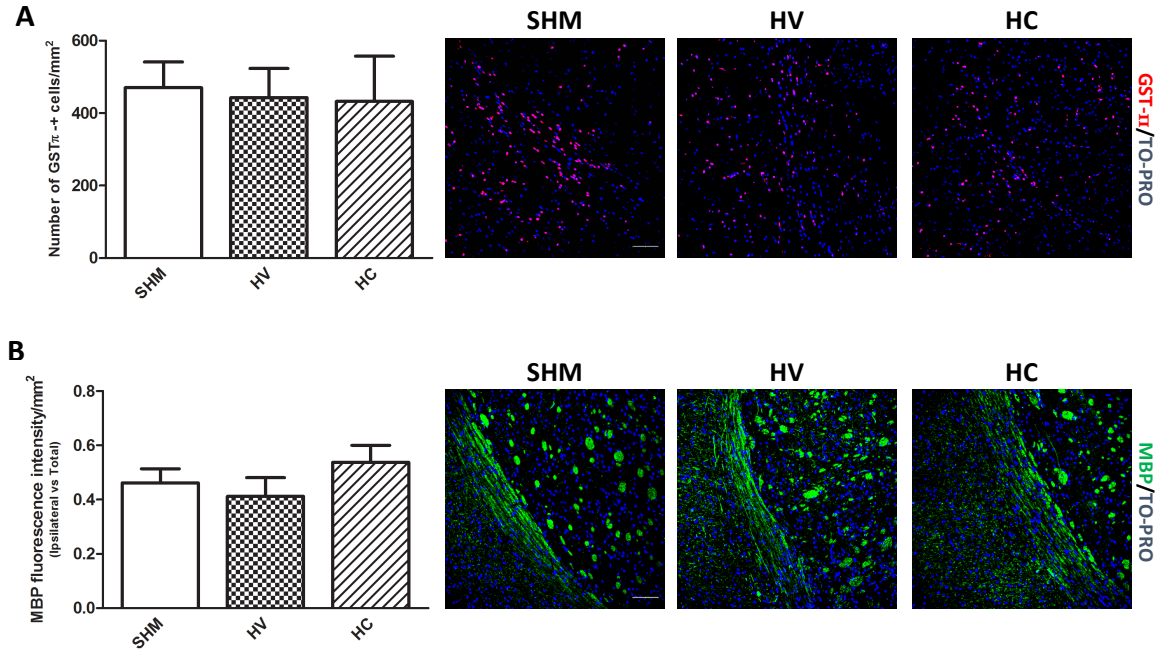


Figure 25. Quantification of mature oligodendrocyte population and myelin intensity signal in white matter. Graphical representation of immunohistochemical studies and representative microphotographs performed in the external capsula of the corpus callosum 30 days after a hypoxic-ischemic (HI) insult induced in P7-10 Wistar rats then receiving vehicle (HV) or cannabidiol (HC), or a similar period in control rats (SHM). **A)** GST-π and **B)** myelin basic protein (MBP) staining. Bars represent mean±SEM. Scale bar: 50µm.

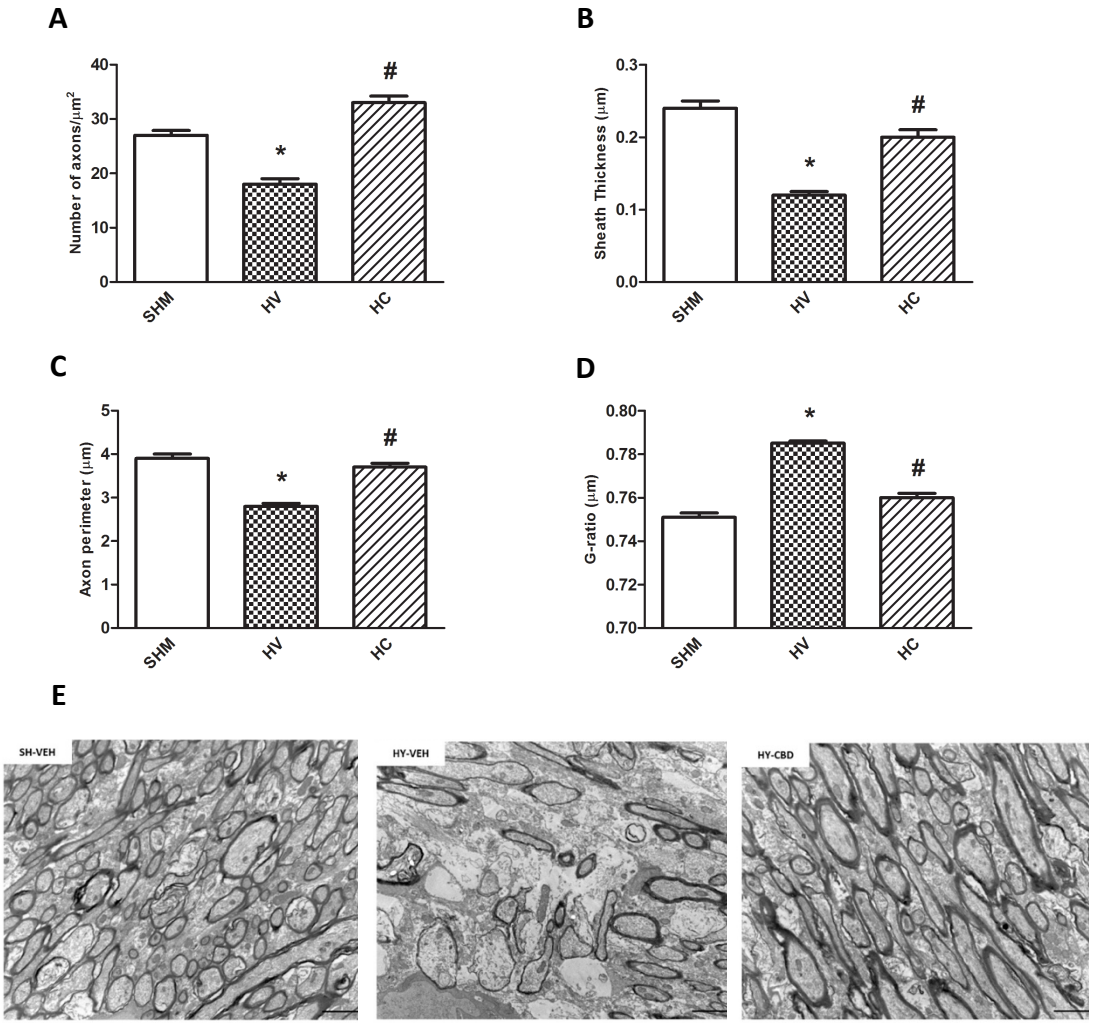


Figure 26. Myelin study in white matter by electron microscopy. Electron microscopy studies performed in the external capsula of the corpus callosum 30 days after a hypoxic-ischemic (HI) insult induced in PND7-10 Wistar rats then receiving vehicle (HV) or cannabidiol (HC), or a similar period in control rats (SHM). **A)** Number of axons; **B)** myelin sheath thickness; **C)** Axon perimeter; **D)** g-ratio; **E)** Representative micrographs. Bars represent mean±SEM. Scale bar: 10μm ** p<0.05 vs SHM; # p<0.05 vs HV by ANOVA*.

2.2 Parietal cortex

In contrast of what was observed in white matter, HI led to a remarkable loss of mature oligodendrocyte one month after the insult (Fig. 27A). Oligodendrocyte reduction was associated with a significant hypomyelination in HV animals as compared to SHM (Fig. 27B). By contrast, CBD administration prevented the loss of GST-π⁺ cells (Fig. 27A) and, consequently, the MBP intensity signal decrease (Fig. 27B).

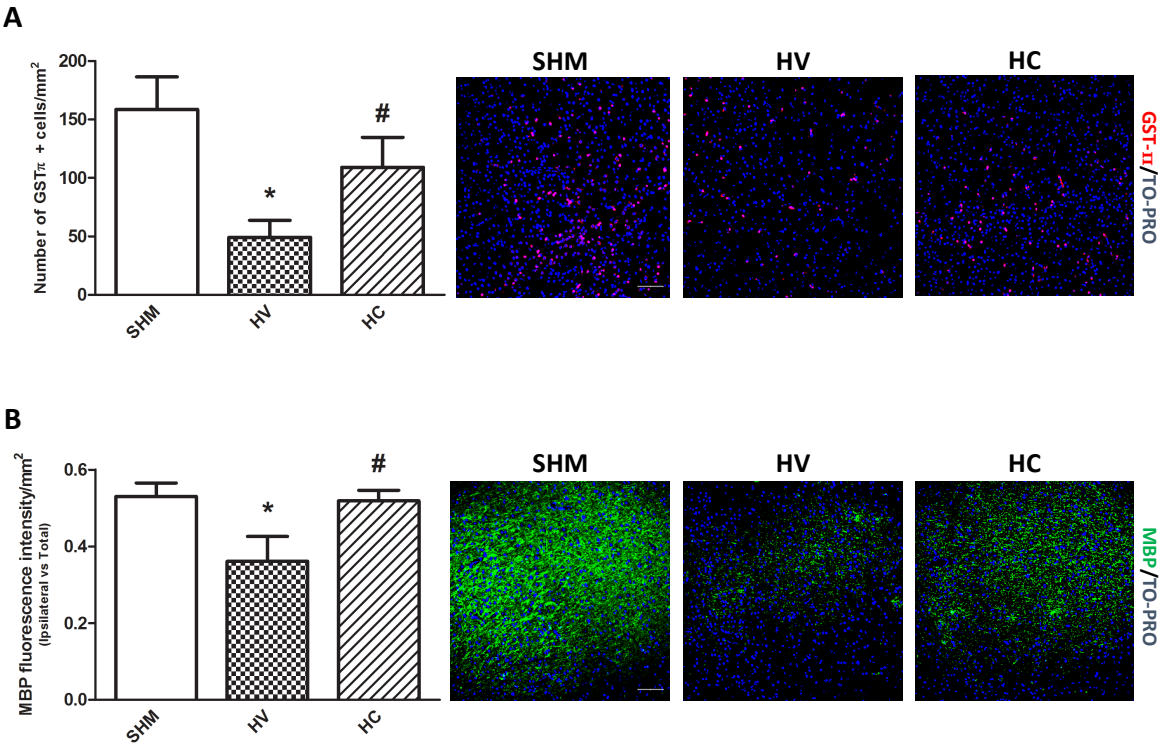


Figure 27. Analysis of mature oligodendrocyte population and myelin staining in cerebral cortex. Graphs and its representative images of immunohistochemical studies performed in the parieto-occipital cortex of PND 37 rats submitted to neonatal HI and that were treated with vehicle (HV) or cannabidiol (HC), while its littermates remained as control (SHM). **A)** GST-π and **B)** myelin basic protein (MBP) staining. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; # p<0.05 vs HV by ANOVA.*

The characterization of this hypomyelination by electron microscopy, showed that HV animals not only had thinner myelin sheaths (Fig. 28B), corroborated by the g-ratio increase (Fig. 28D), but also a remarkable reduction of axons (Fig. 28A). No differences in axon perimeter were observed in cortex (Fig. 28C). CBD prevented both axon loss (Fig. 28A) and myelin sheath thickness reduction (Fig. 28B), with g-ratio values similar to those of SHM (Fig. 28D).

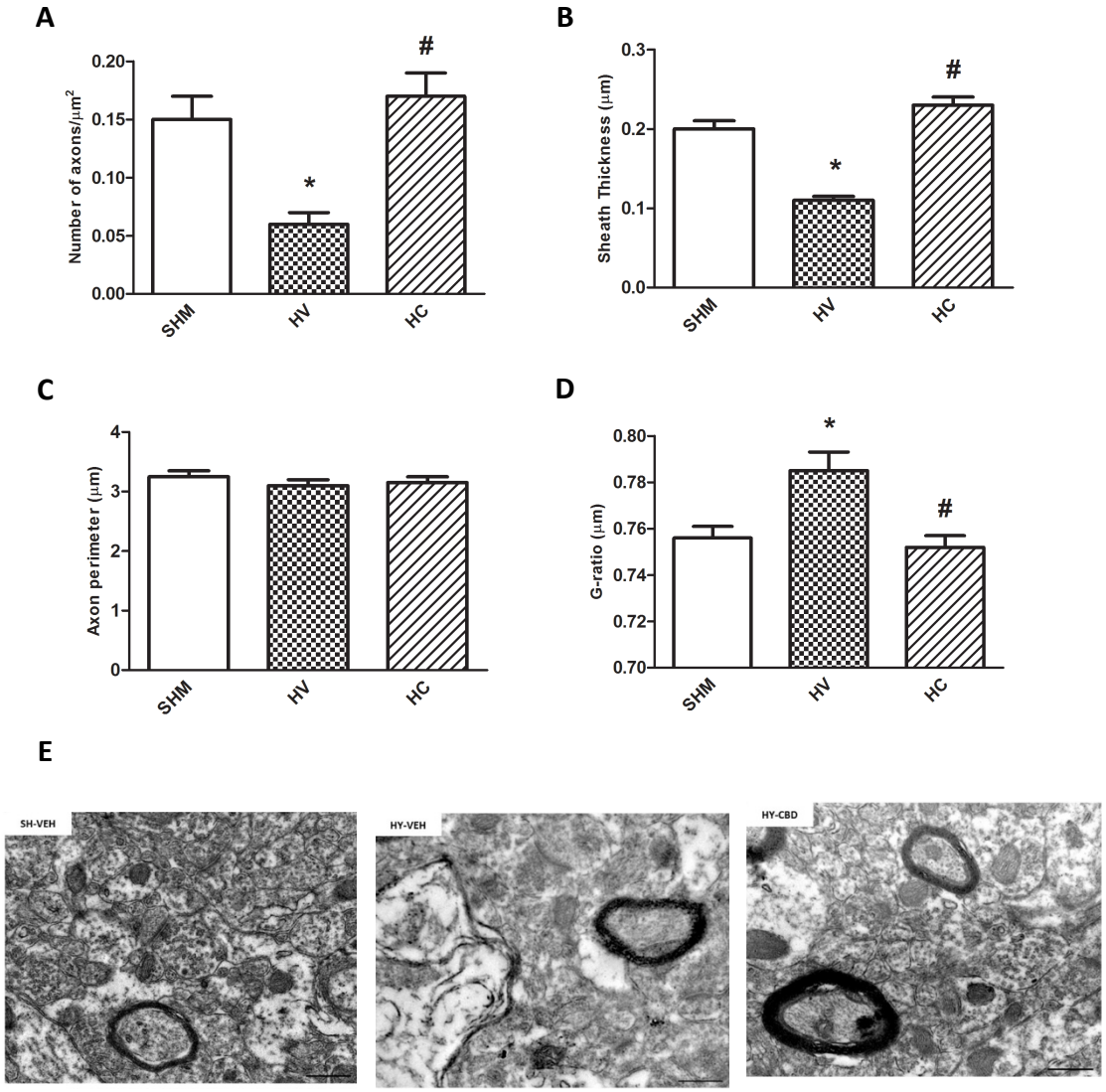


Figure 28. Myelin characterization in cerebral cortex by electron microscopy. Myelin analysis performed in the parieto-occipital cortex of rats one month after they were submitted to a neonatal HI insult and received vehicle (HV) or cannabidiol (HC), or were left as controls (SHM). Graphs represents the **A)** Number of axons; **B)** myelin sheath thickness; **C)** axon perimeter; **D)** g-ratio; **E)** representative micrographs. Representative microphotographs are observed in the left. Bars represent mean±SEM. Scale bar: Scale bar: 10µm. ** p<0.05 vs SHM; # p<0.05 vs HV), by ANOVA*.

2.3 Functional studies

HI damage led to hemiparesis as illustrated by the preference for the use of the ipsilateral forepaw in the CRT (Fig. 29A). HV group also had a significant cognitive disability as studied in the memory test (NOR), with HV animals displaying similar time exploring both the new and the old object (Fig. 29B). When this motor and cognitive impairment was compared with the MBP signal intensity in both White and Grey Matter, a positive correlation was observed between White Matter-MBP and hemiparesis, i.e. animals with poorer results in CRT test also had lower

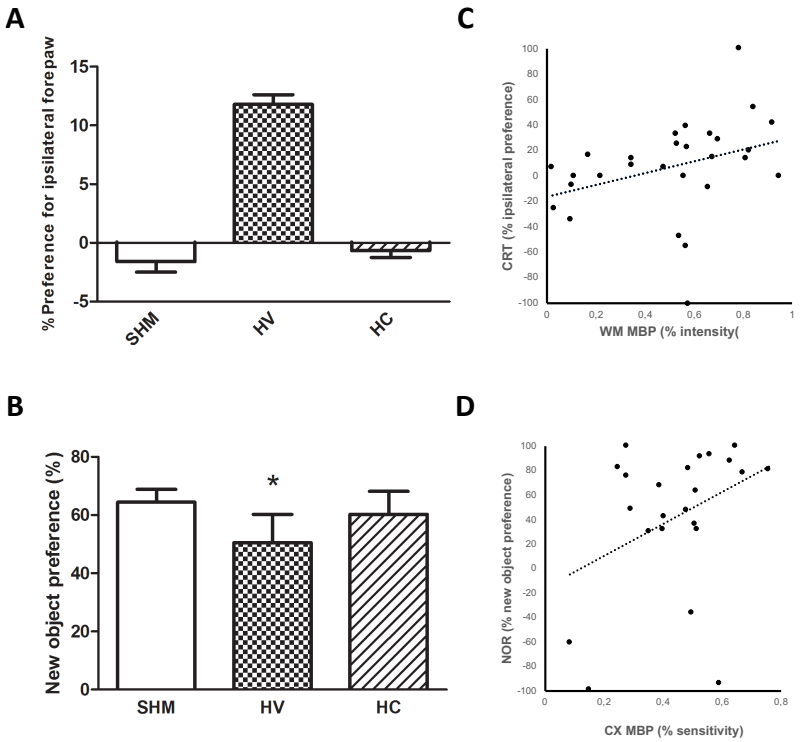


Figure 29. Relationship between immunohistochemical and neurobehavioral studies. Neurobehavioral studies and its linear correlation with MBP staining intensity performed 30 days after a HI insult. **A)** Preference for the use of the ipsilateral paw in the cylinder rearing test (CRT); **B)** Time percentage of the time exploring the new object versus total exploring time in the NOR test; **C)** Correlation between hemiparesis as assessed by the CRT and MBP intensity in white matter (Spearman's correlation: R= 0.396, t= 2.58, p=0.013. **D)** Correlation between memory impairment as assessed by NOR and MBP intensity in cortex; Spearman's correlation: R= 0.443, t= 2.57, p=0.015). ** p<0.05 vs SHM by ANOVA.*

MBP signal in corpus callosum (Fig. 29C). At the same time, memory impairment was positively correlated with cortex hypomyelination (Fig. 29D).

Although no changes could be observed between HV and HC in the correlation, CBD administration was able to hamper both hemiparesis and cognitive impairment-HI induced (Fig. 29A and B).

2.5 Summary

To summarize the results of this goal, HI induced a significant hypomyelination and axonopathy observable one month after the damage. Myelination impairment was observed in both corpus callosum and parieto-temporal cortex, although only in the cerebral cortex a reduction in the population of mature oligodendrocyte could be observed. Remarkably, hypomyelination correlated with the long-term motor and cognitive deficits detected. By contrast, CBD administration prevented mature oligodendrocyte decrement in cortex as well as hypomyelination and axonopathy in both areas. In agreement with those results, HC group did not show the motor and cognitive impairment induced by HI.

3. ASSESSING IF CBD HYPOMYELINATION PREVENTION IS RELATED WITH BRAIN CELL PROLIFERATION ACTIVATION AND GLIAL RESPONSE MODULATION

After demonstrating the prevention of hypomyelination by CBD, the next step was to elucidate how that effect of CBD could be achieved. Since it is known that HI triggers a proliferative response in brain in order to substitute the cell loss (Brégère et al., 2017; Plane et al., 2004; Segovia et al., 2008), we started by studying the proliferative response induced by HI and its modification by CBD administration.

3.1 Cell proliferative response and cell death

First at all, proliferating cells rates were studied on both corpus callosum and parieto-occipital cortex, 24 hours and seven days after HI. No differences were observed at 24 hours in any region (Fig. 30). Seven days after the insult HC group showed increased $Ki67^+$ cell population in White Matter as compared to SHM, an effect not observed in HV (Fig. 30A). By contrast, in parietal cortex the HI-induced potent proliferative response was not statistically different in HV and HC, although such response tended to be lower in HC group seven days after the insult (Fig. 30B). This proliferative response was associated with an increase of mature and proBDNF protein levels seven days after the HI insult with no significative differences between HV and HC, although proBDNF levels tended to be lower in HC group (Fig. 31A) . No changes were detected in the protein levels of GDNF and IGF-1 (Fig. 31B and D).

Then, proliferative response was studied in the SVZ (Fig. 32A). HI led to a quick proliferative response in the SVZ, observable just one day after damage in both HI groups. Seven days after HI, HV showed a non-significant reduction of $Ki67^+$ cell density in SVZ, an effect not detected in HC group (Fig. 32B).

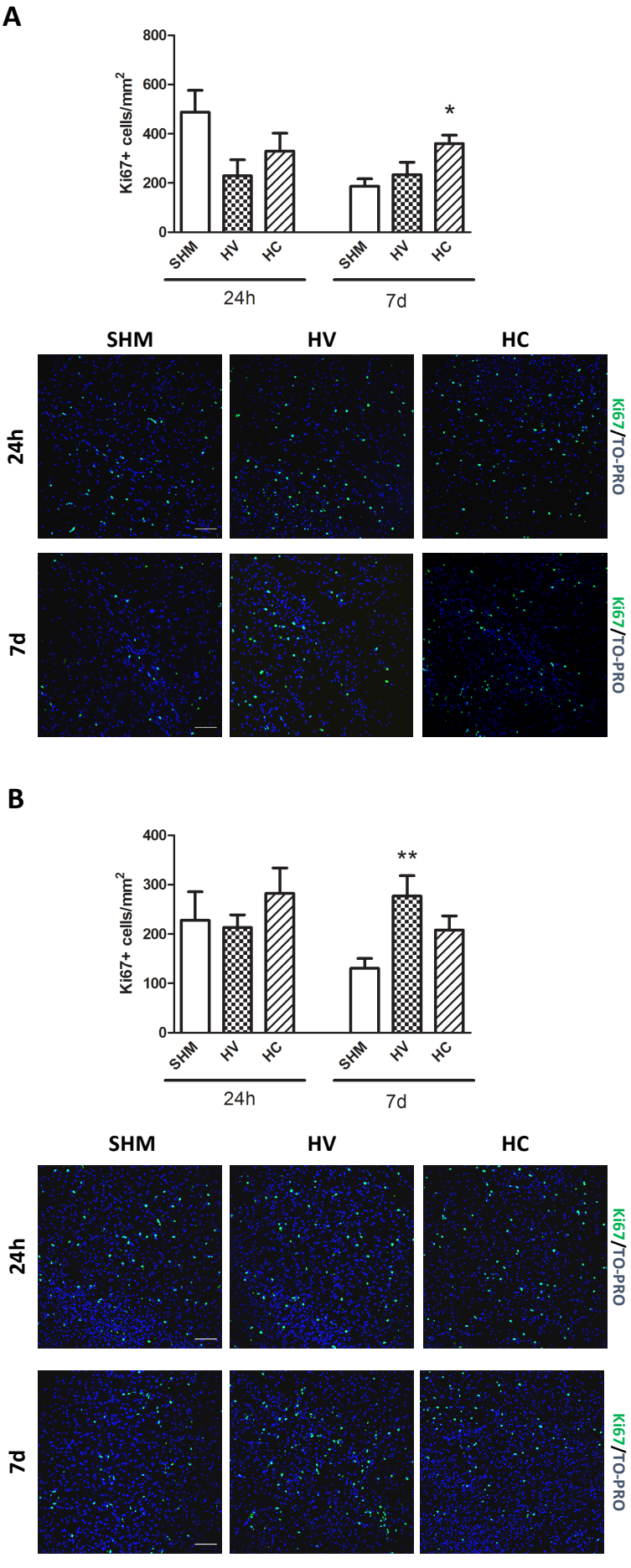


Figure 30. Proliferation characterization in corpus callosum and cerebral cortex. Representative microphotographs and graphical representation of proliferation studies performed 24 hours and 7 days after a neonatal HI insult induced Wistar rat pups which were then treated with vehicle (HV) or cannabidiol (HC), and its control littermates (SHM). The regions represented are **A)** external capsule of corpus callosum and **B)** parieto-occipital cerebral cortex. Scale bar: 50µm.
*** p<0.01 vs SHM by ANOVA.*

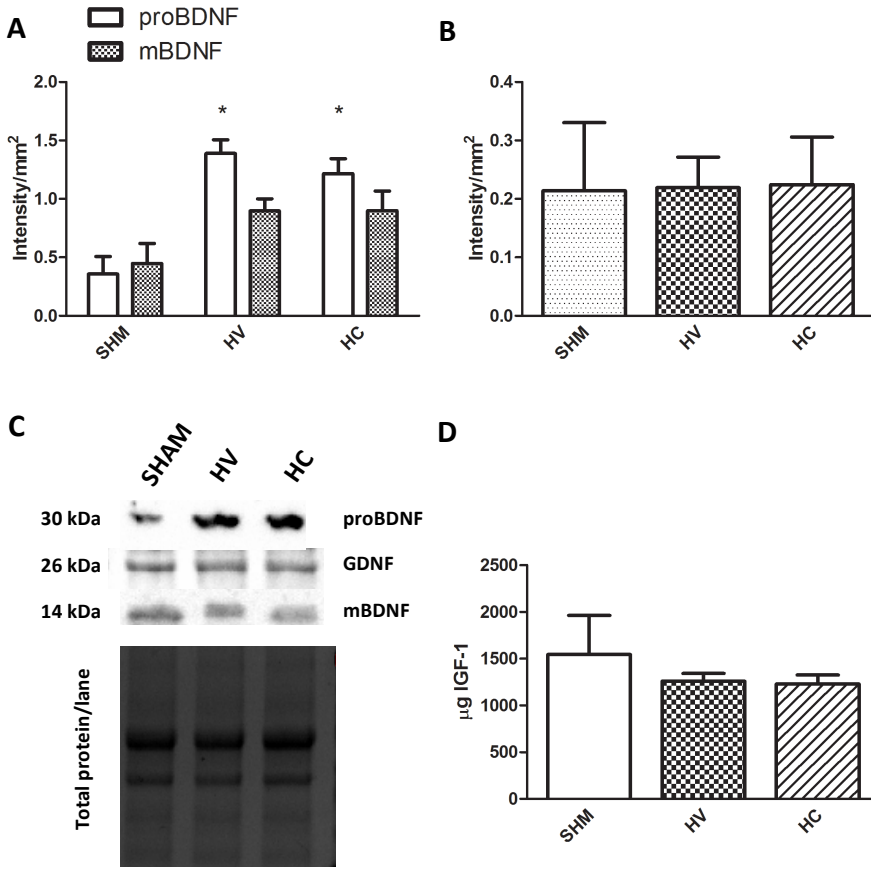
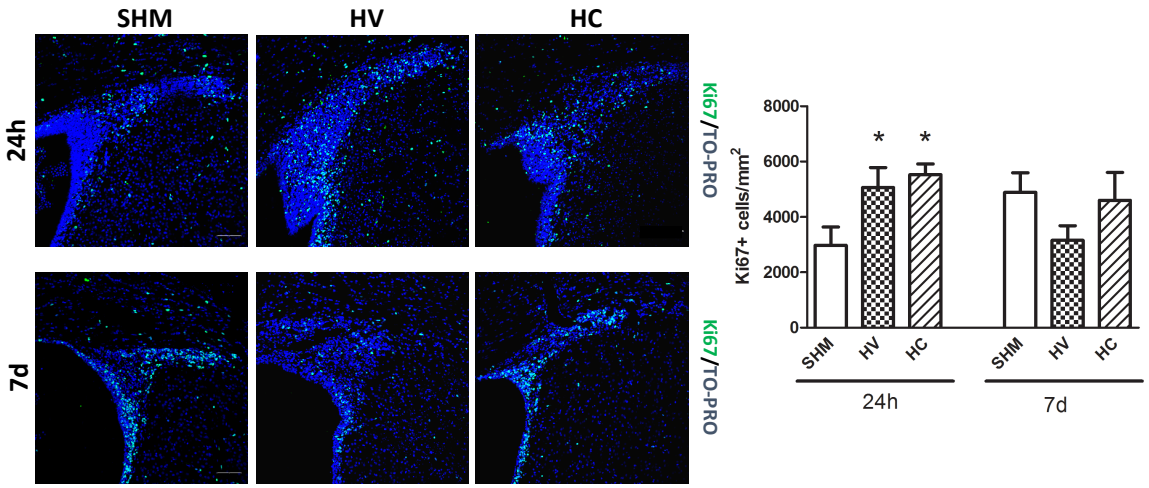


Figure 31. Neurotrophins quantification at cerebral cortex. Western blot studies performed in cerebral cortex of Wistar rats one week after a neonatal HI insult. **A)** Quantitative determination of the precursor (proBDNF) and mature (mBDNF) isoforms of brain derived neurotrophic factor (BDNF) expression; **B)** Quantitative determination of glia derived neurotrophic factor (GDNF) expression; **C)** Representative images of western blot; **D)** Quantification of insulin growth factor-1. Bars represent mean±SEM* *p<0.05 vs SHM by ANOVA.*

Figure 32. Ki67 immunohistochemical study in SVZ. Ki67-positive cells quantification performed in SVZ 24 hours and 1 week after a neonatal hypoxic-ischemic insult induced in PND7-10 Wistar rats then receiving vehicle (HV) or cannabidiol (HC), or a similar period in control rats (SHM). Representative images (right) and graphical representation of Ki67-positive cells (left). Scale bar: 50µm. ** p<0.05 vs SHM by ANOVA.*



Besides, HI led to increased cell death in parietal cortex, as observed by TUNEL staining (Fig. 33A). In this case no significative differences were observed between HC and HV groups (Fig. 33B).

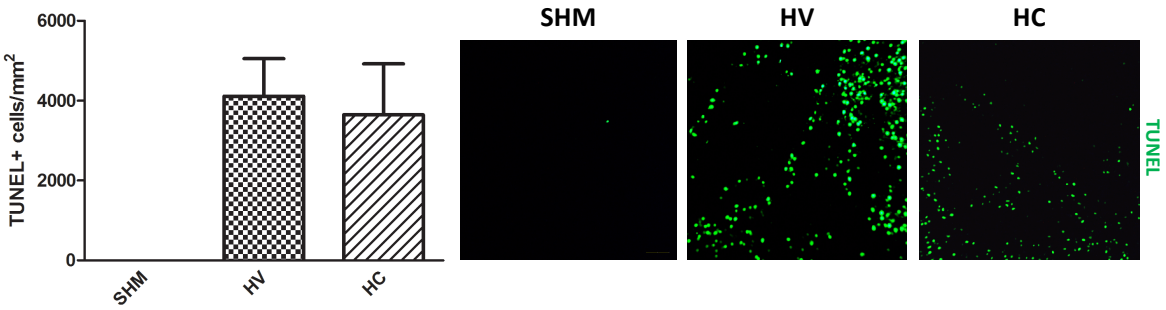


Figure 33. Cell death quantification in parieto-occipital cortex. Staining for TUNEL-positive cells in the cerebral cortex of rat pups seven days after a HI insult and its treatment with vehicle (HV) or CBD (HC). Graphical representation of TUNEL-positive cells (left) and illustrative microphotrographs (right). Bars represent mean±SEM.

3.2 Oligodendrocyte response

For a better understanding of CBD effects on hypomyelination induced by HI, oligodendrocyte population was studied by immunohistochemistry in the regions involved: cortex, corpus callosum and SVZ. When SVZ was analyzed, no differences among groups in $Olig2^+$ cell quantification was observed at any time (Fig. 34A). $SOX10^+$ cell density was decreased 24 hours after the insult. Seven days after the insult $SOX10^+$ cell density was restored (Fig. 34B) with even a non-significant increase in both HI groups (Fig. 34B). By contrast, in corpus callosum a significant decrease of both Olig2 (Fig. 35A) and SOX10 (Fig. 35B) positive cell population was observed 24 hours after damage with no differences between HV y HC. $Olig2^+$ and $SOX10^+$ cell density was restored one week after HI (Fig. 35) with a non-significant reduction of $SOX10^+$ cell density in HC (Fig. 35B).

The analysis of oligodendrocyte linage cells on cortex proved that, similarly to that observed in corpus callosum, HI induced a rapid decrease of Olig2 (Fig. 36A) and SOX10 (Fig. 36B) positive cell population that was restored one week after the insult. While CBD had no effect 24 hours after HI, an increase of $Olig2^+$ cells was observed one week later (Fig. 36A). However, no difference was seen in $SOX10^+$ cell density, with both HV and HC showing similar levels than SHM at that timepoint (Fig. 36B).

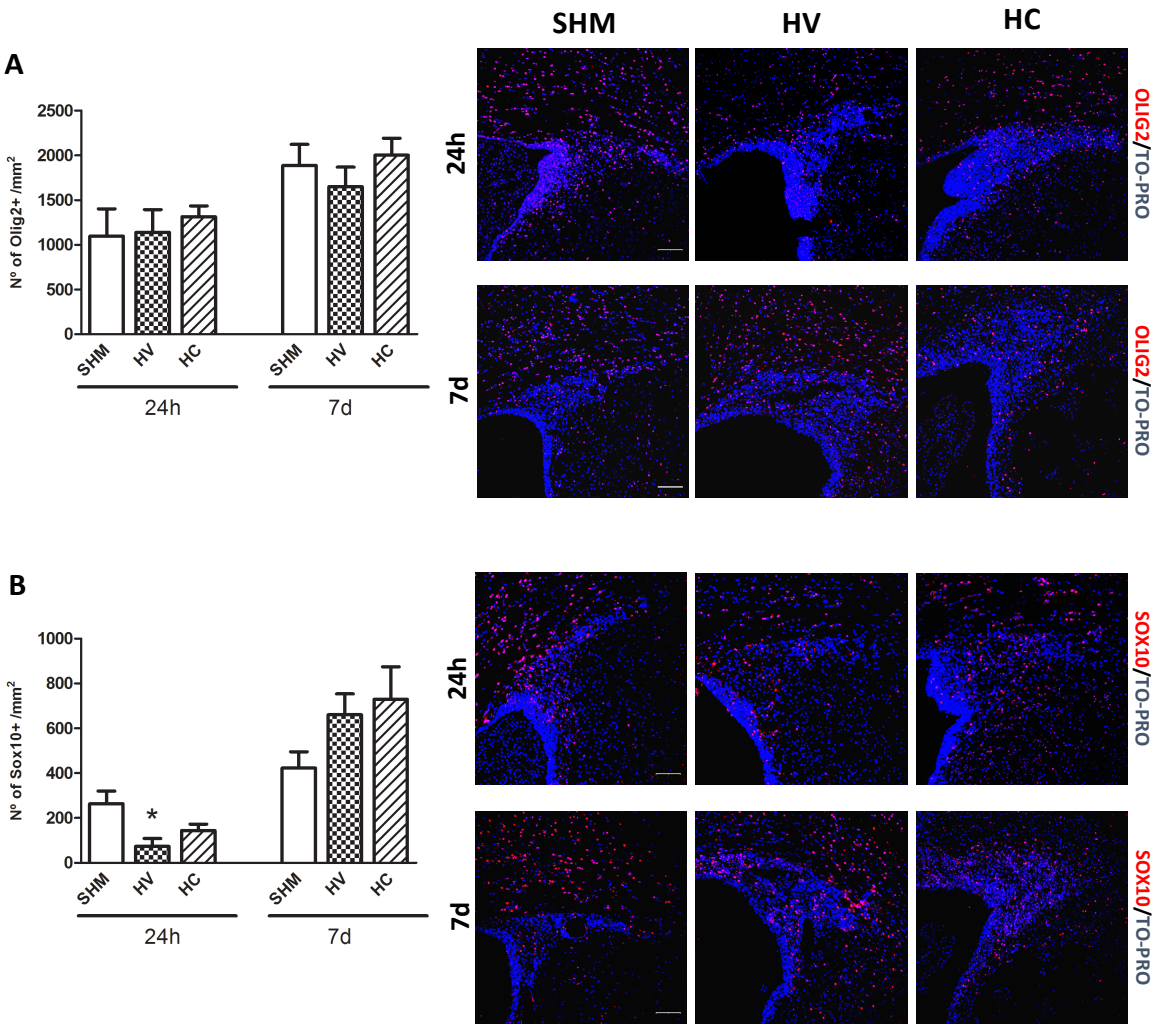
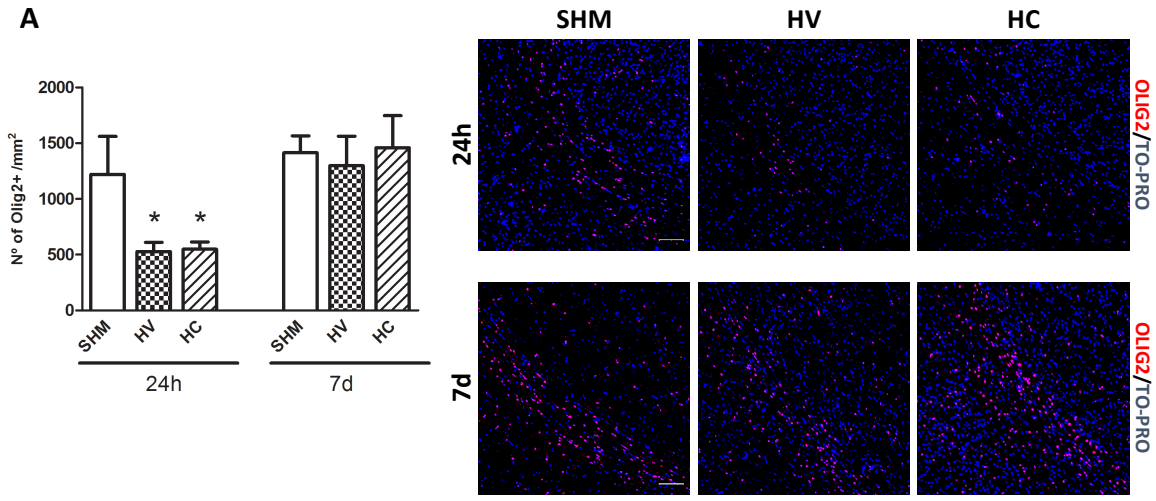


Figure 34. Quantification of oligodendrocyte lineage population in the subventricular zone. Illustrative microphotographs and graphs of the immunohistochemical studies performed 24 hours and 7 days after a HI in the different groups. **A)** Olig2 and **B)** SOX10 staining in corpus callosum. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM by ANOVA.*



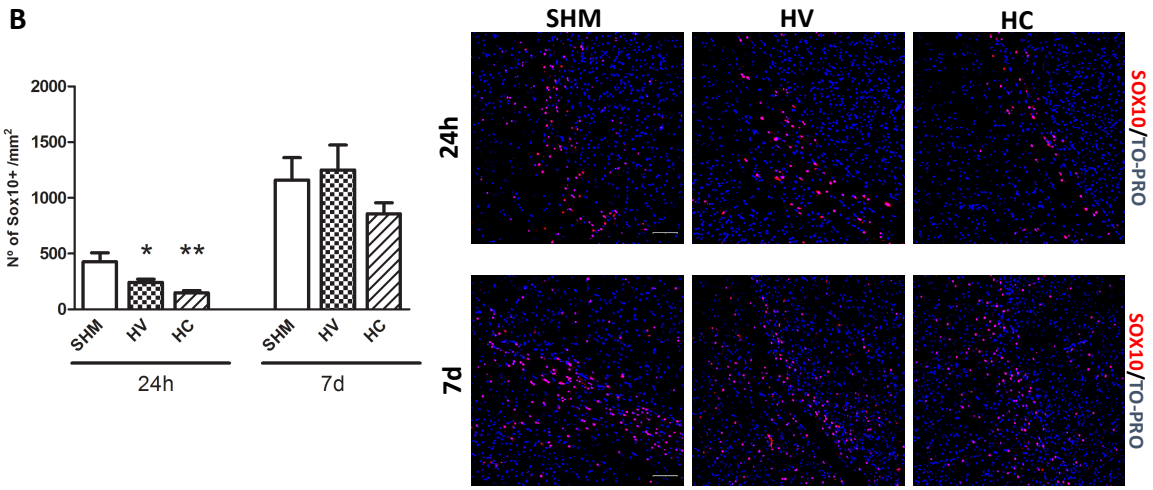


Figure 35. Quantification of oligodendrocyte lineage population in white matter. Immunohistochemical quantification of oligodendrocyte lineage were performed 24 hours and 7 days after a HI in the different groups. Illustrative images could be also observed at the right of the figure. **A)** Olig2 and **B)** SOX10. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; ** p<0.01 vs SHM by ANOVA.*

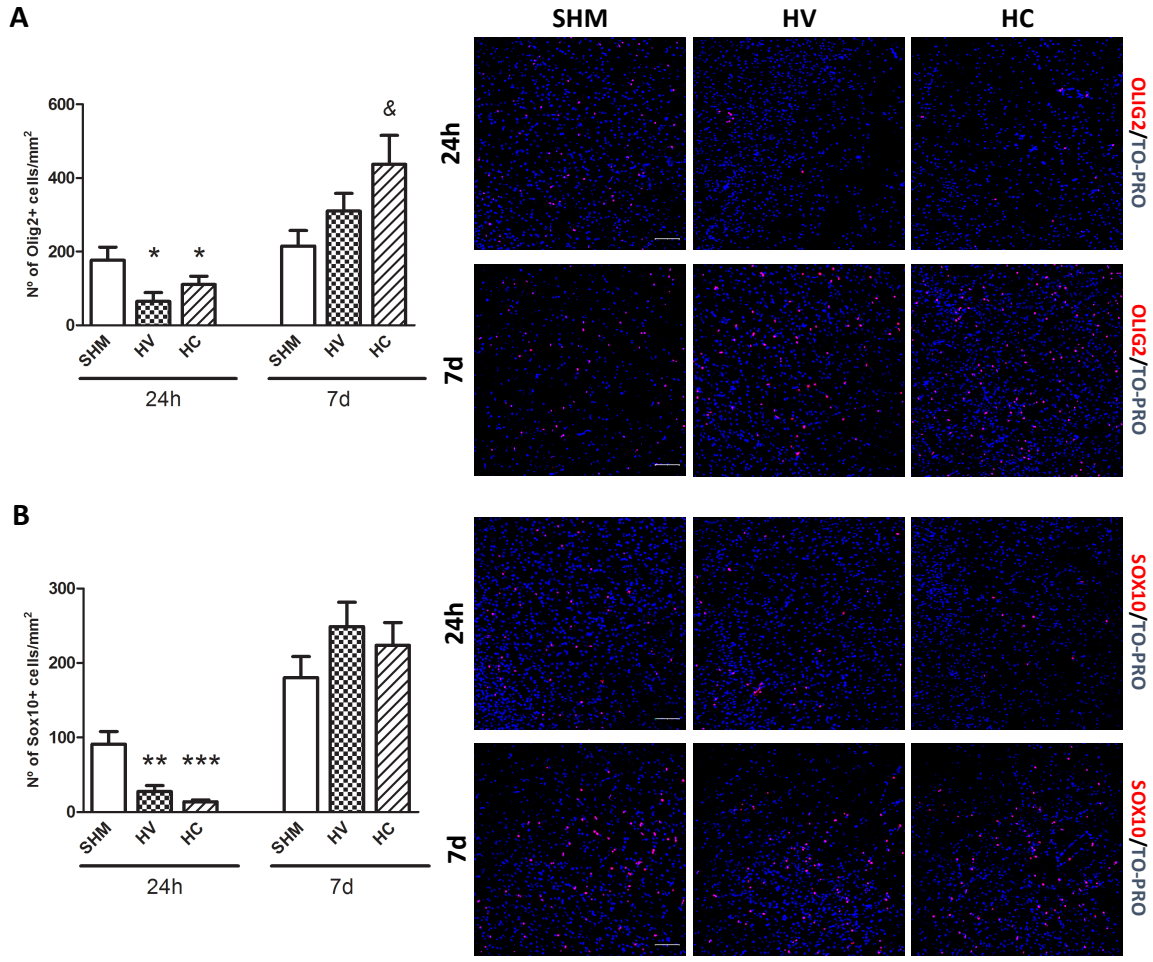


Figure 36. Analysis of oligodendrocyte lineage population in parieto-occipital cortex. Representative microphotographs (right) and graphical representation (left) of Olig2 and Sox10 immunohistochemical studies in parietal-occipital cortex of rat brain 24 hours and 7 days after damage. **A)** Olig2 and **B)** SOX10 staining. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM 24h ; ** p<0.01 vs SHM 24h ; *** p<0.001vs SHM 24h ; & p<0.05 vs SHM7d by ANOVA*

Since cell density changes were mainly observed in cortex subsequent studies were focused in this region. To further understand the discrepancies between Olig2 and SOX10 analysis different experiments were performed. First, BrdU was injected at fourth, fifth and sixth day after the insult and then pups were sacrificed at the seventh day. Labelling the new cells developed during that period with different oligodendrocyte markers would facilitate the characterization of the proliferative and maturative response induced by HI and how it could have been modified by CBD. The first step was to look for some differences in the number of new cells produced between the fourth and the sixth days after the insult. Surprisingly, the HI insult did not result in a different density of newly formed cells as studied one week after the insult, no matter the treatment with VEH or CBD (Fig. 37A). The same lack of statistically significant differences was observed in the density of newly formed ($BrdU^+$) $Olig2^+$ or $SOX10^+$ cells (Fig. 37B and 37C), although a non-statistically significant increase of $BrdU^+/Olig2^+$ cells was observed in HC animals (Fig. 37B) whereas a non-statistically significant decrease of $BrdU^+/SOX10^+$ cells was observed in HV animals.

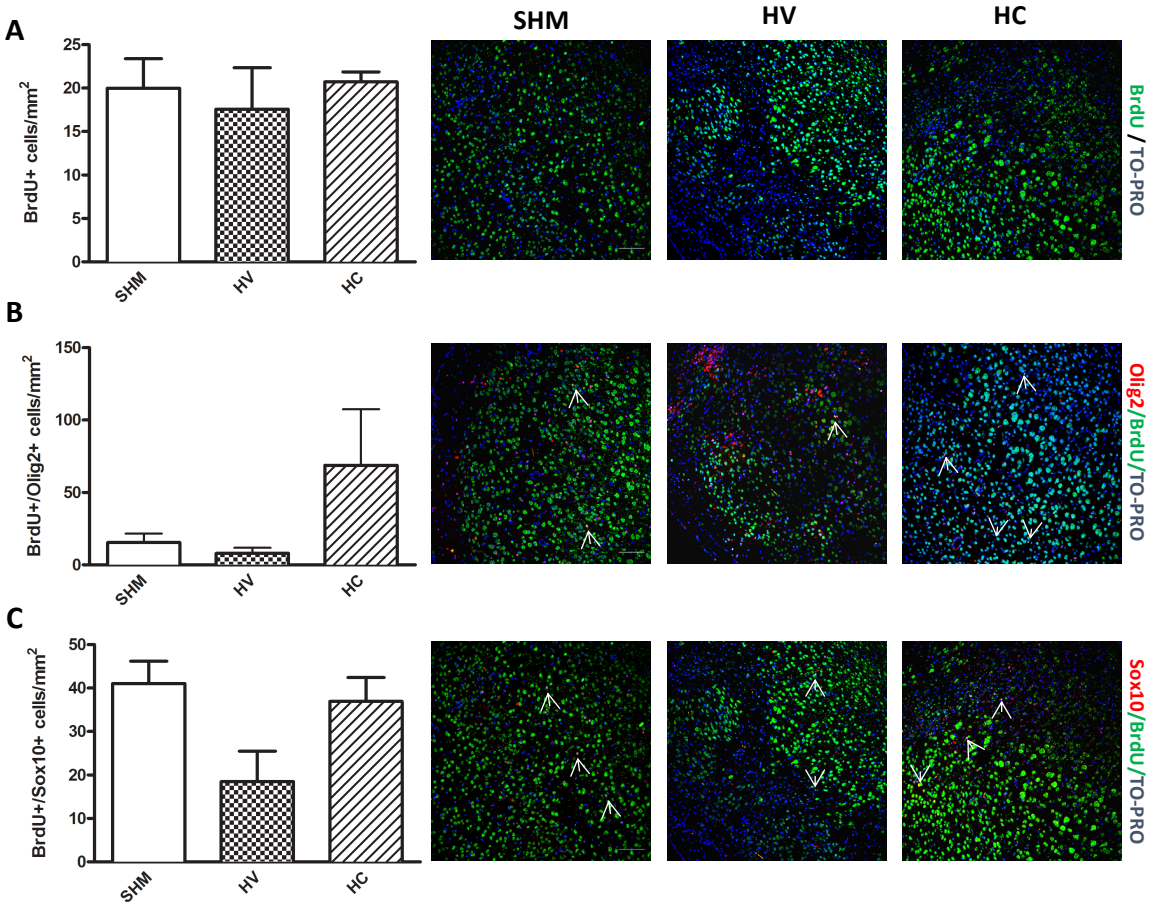


Figure 37. BrdU study in cerebral cortex seven days after damage. Representative microphotographs and graphs of BrdU analysis and colocalization with oligodendrocyte markers in parieto-occipital cortex one week after damage. **A)** BrdU-positive cell quantification; **B)** $BrdU^+$ /$Olig2^+$quantification; **C)** $BrdU^+$ /$SOX10^+$ quantification. Scale bar: 50µm.

In order to get more insights into those differences, co-localization of Ki67 and Olig2 marker was analysed. Since Olig2$^+$ cells could also label astrocytes, the co-localization of GFAP and Olig2 markers was also performed. In agreement with the aforementioned results from BrdU studies, no differences were detected among groups after double-immunostaining studies were conducted with Olig2 and Ki67 antibodies (Fig. 38A). By contrast, a dramatic increase in the number of cells expressing both GFAP and Olig2 markers were found in HV group (Fig. 38B). That effect was reversed by CBD so that HC rats showed similar colocalization rates to SHM (Fig. 38B).

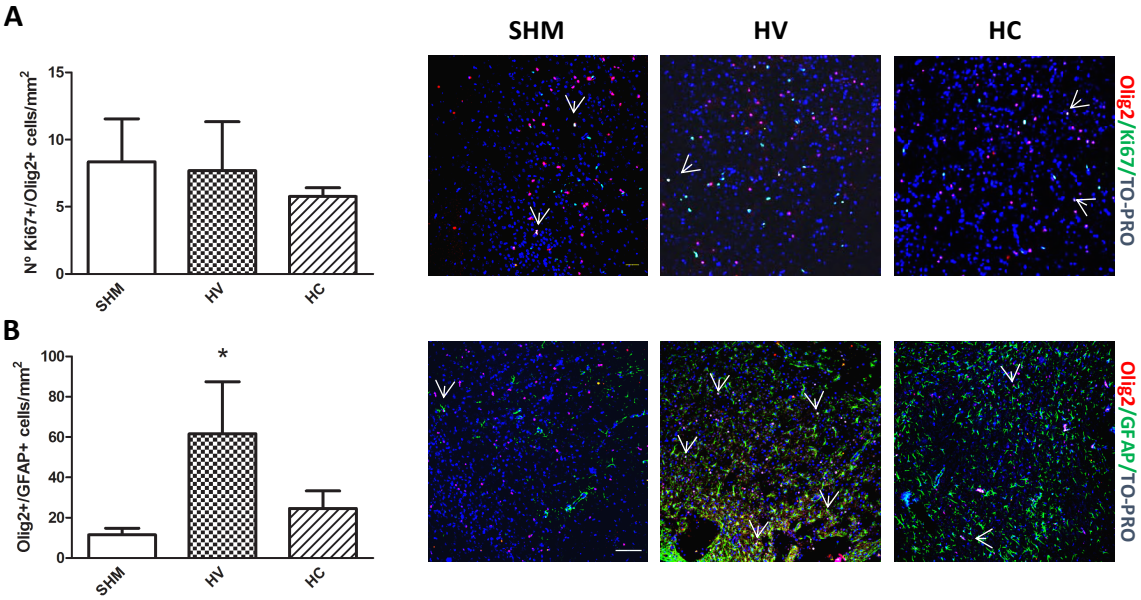


Figure 38. Study of Olig2 colocalizations in cerebral cortex. Representative microphotographs of Olig2 colocalization with the proliferative marker Ki67 or the astroglial marker GFAP, and its graphical quantification in parieto-occipital cortex one week after damage. **A)** Olig2$^+$/Ki67$^+$; **B)** Olig2$^+$/GFAP$^+$. Scale bar: 50µm. ** p<0.05 vs SHM by ANOVA.*

The analysis of BrdU$^+$/NeuroD$^+$ cells showed a non-significant decrease in HV group as compared to SHM (Fig. 39A), in contrast with the increased proliferation observed by Ki67 in HV group (Fig. 30B). Further analysis confirmed that HI induced a significant reduction of the new immature neurons seven days after damage while CBD administration led to a newly formed immature cell density similar to that of SHM at that timepoint (Fig. 39B).

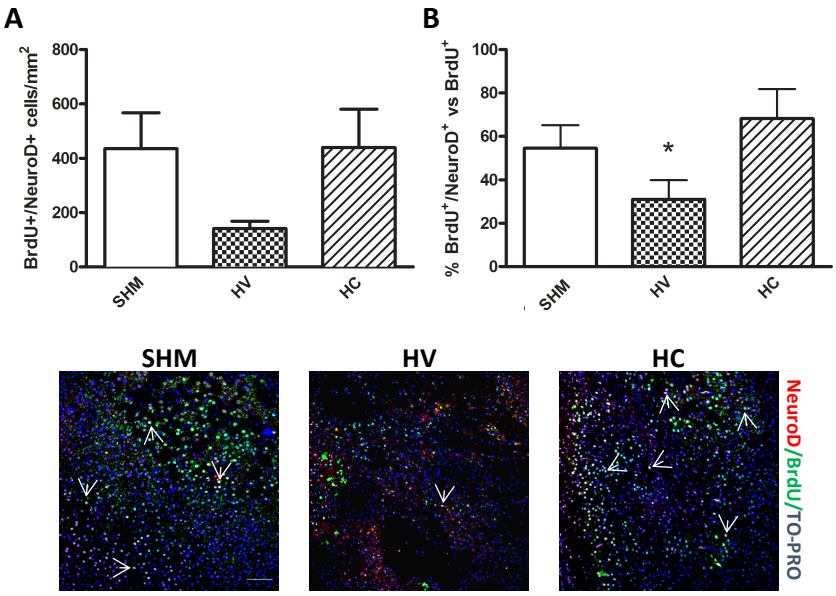


Figure 39. BrdU⁺/NeuroD⁺ colocalization analysis in cerebral cortex one week after HI. Representative images and graphs of BrdU colocalization with immature neuronal markers NeuroD in parieto-occipital cortex seven days after HI. **A)** Number of cells which co-express both markers; **B)** Percentage of BrdU⁺ cells which are also positive for NeuroD marker. Scale bar: 50µm. ** p<0.05 vs SHM by ANOVA*

3.3 Astroglial response

In order to further study if the increment of Olig2⁺/GFAP⁺ cell proliferation in HV rats was associated with an enhanced gliotic response, astrocytes and microglial population were analyzed separately. As expected, a noteworthy rise of GFAP⁺ cells was observed in HV animals as compared to SHM, an effect reduced by CBD administration (Fig. 40A). That enhancement was associated to a gain of GFAP fluorescence intensity (Fig. 40B), which corresponds with an active astrocyte (Cengiz et al., 2014; Diaz et al., 2017). HC group reduced astrocyte activation, with a non-significant reduction in GFAP fluorescence intensity (Fig. 40C).

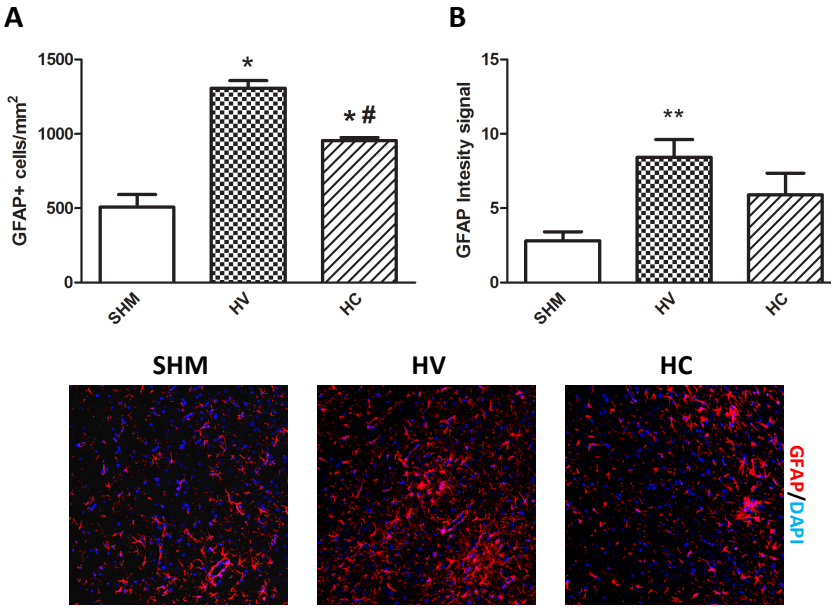


Figure 40. Astrogliosis analysis in parieto-occipital cortex. Graphical representation (up) and illustrative images (down) of immunohistochemical studies of the astroglial marker GFAP performed in the parieto-occipital cortex 7 days after a HI insult. **A)** GFAP- positive cells quantification; **B)** GFAP intensity signal quantification. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; ** p<0.01 vs SHM; # p<0.05 vs HV by ANOVA.*

3.4 Microglial response

HI insult led a remarkable microgliotic response that was not affected by CBD administration, (Fig. 41A). Regarding microglia morphology there were no statistically significant differences in microglial cell soma size among the different groups (Fig. 41B), although in HV animals microglial cells showed shorter ramifications and an ameboid shape (Fig. 41C), corresponding with an active microglia phenotype. HC and SHM groups had similar microglia soma size and ramifications lengths (Fig. 41B and C).

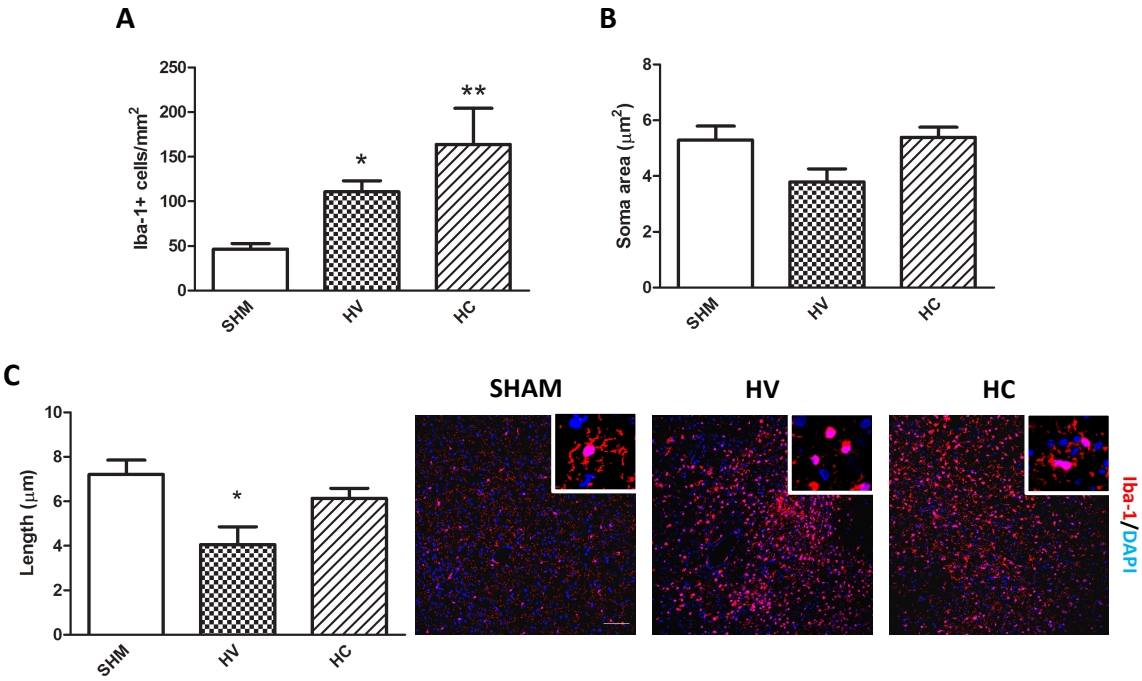


Figure 41. Microgliosis study in parieto-occipital cortex. Characterization of microglial response by cell quantification and morphological studies performed seven days after damage. Illustrative microphotographs of microglia population with cell augmentation of the main cell morphology found are represented at bottom right. **A)** Iba1- positive cells quantification; **B)** Soma area of Iba-1 positive cells; **C)** Average of dendrite length of Iba-1 positive cells. Bars represent mean±SEM. Scale bar: 50µm. ** p<0.05 vs SHM; ** p<0.01 vs SHM by ANOVA*

3.5 "Deadly triad" analysis

To get more insights into the gliotic response, TNFα levels were analysed by western blot. As predicted, brain TNFα concentration was increased in HV group one week after the HI insult (Fig. 42A). Such increase was prevented by CBD administration, with TNFα protein concentration reaching similar levels to those observed in SHM (Fig. 42A).

Due to the potent CBD antioxidant effect, HI-induced increase of oxidative stress was studied in brain samples by quantifying both carbonyl groups in oxidized protein by western-blot and glutathione by H^+-MRS. By using both techniques a dramatic increase of oxidative stress markers was observed in HV group one week after damage, as expected (Fig. 42B and C). CBD administration fully prevented the HI-induced increase of oxidative stress (Fig. 42B and C).

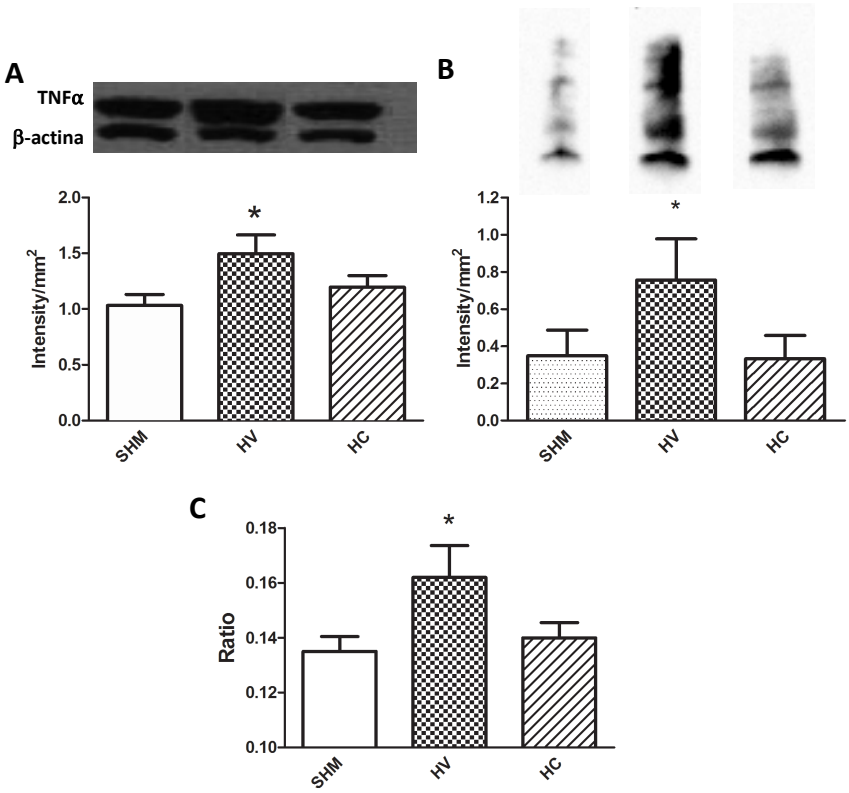


Figure 42. Inflammation and oxidative stress analysis in parieto-occipital cortex. Western and H^+-RMS studies performed in the ipsilateral cerebral cortex one week after HI. **A)** TNFα quantification by western-blot and representative bands of TNFα (50KDa) and β-actina (42KDa) ; **B)** Graphical representation of OxyBlot analysis and illustrative lanes of carbonyl groups in oxidize; **C)** Glutathione (GSH/Cr) analyzed by H^+-RMS . Bars represent mean±SEM. ** p<0.05 vs SHM by ANOVA.*

The third axis of the "deadly triad" is excitotoxicity. An increased concentration of glutamate was observed by H^+-MRS one week after the HI insult in HV animals (Fig. 43), an effect blunted by CBD administration.

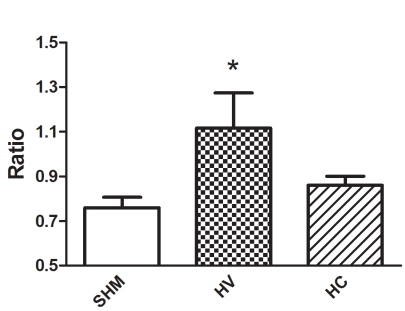


Figure 43. Glutamate quantification in parieto-occipital cortex. Glutamate quantification by H^+-RMS in the parieto-occipital cortex seven days after HI insult. Bars represent mean±SEM. ** p<0.05 vs SHM by ANOVA.*

3.6 Summary

To sum up the most important results of this goal, HI induced early death of oligodendrocytes in both areas of damage resulting in reduced cell density; oligodendrocyte density was restored seven days after the insult. Besides, CBD administration led to increased $Olig2^+$ population in cortex at that timepoint; BrdU studies showed that HC group had more new cells $Olig2^+$ produced between the fourth and the sixth day after the insult. Furthermore, CBD prevented the decrease of $SOX10^+$ cell density observed at that timepoint. In agreement with these results, a more actively differentiation of $Olig2^+$ population into astroglial lineage was observed in HV but not in HC group. This was also observed after studying astrocyte and microglial cells. In this case CBD administration decreased both astrogliosis and microgliosis in a manner linked to the modulation of oxidative stress and inflammation. Finally, CBD administration led to reduced excitotoxicity in cortex seven days after the insult.

4.INVOLVEMENT OF CB_2 RECEPTOR ACTIVATION IN CBD PROTECTIVE EFFECTS IN A NEWBORN RAT MODEL OF HYPOXIC-ISCHEMIC BRAIN DAMAGE

We then aimed to characterize if CB_2 receptors could be involved in CBD neuroprotection. We chose CB_2 since astro- and microglia modulation seemed to be a key part of such effect, and both are known to express CB_2 in a pathological or stressful situations (Espejo-Porras et al., 2018; Fernández-Ruiz et al., 2007; López et al., 2018; Robertson et al., 2017; Sagredo et al., 2009; Tolón et al., 2009). Moreover, the involvement of CB_2 activation in CBD protective effect in a piglet model of HI was previously observed by our group (Pazos et al., 2013).

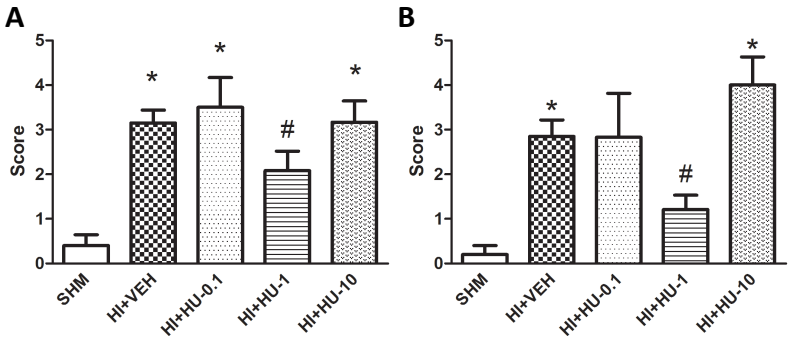


Figure 44. HU-308 dose optimization in a HI model in newborn rat. Semi-quantitative score of cell death by Nissl staining performed in: **A)** parieto-occipital cortex and **B)** CA1 of hippocampus. Bars represent mean±SEM. ** p<0.05 vs SHM; # p<0.05 vs HV) by ANOVA.*

The first step was optimizing the dose of the CB_2 agonist used, HU-308, in the HI model in newborn rats. From the three doses tested of HU-308 only 1mg/Kg reduced the histological damage induced by HI in parietal

cortex (Fig. 44A) and hippocampus (Fig. 44B), while 0.1 and 10mg/Kg did not show any protective effect. Thereby, 1mg/kg was the dose selected for the experiment.

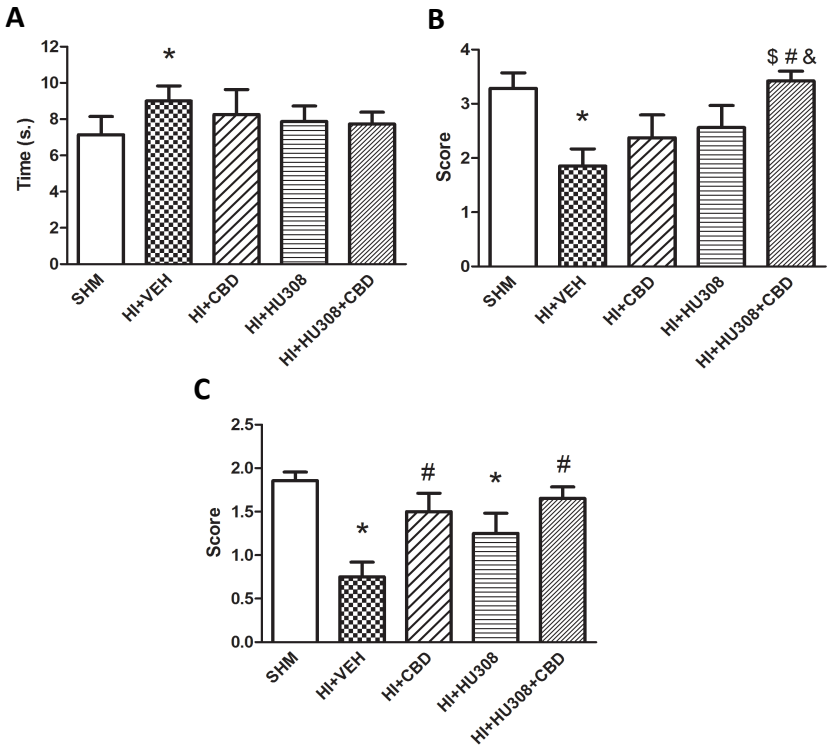


Figure 45. Motor reflex evaluation of CBD and HU-308 prevention of HI-induced motor impairment. Motor reflexes tests conducted in Wistar rats one week after they were submitted at PND7-9 to sham operation (SHM) or to HI, and were treated whether with CBD, VEH or with the CB_2 agonist HU-308. **A)** Negative geotaxis; **B)** Grip reflex and **C)** Grasp reflex. Bars represent mean±SEM. ** p<0.05 vs its SHM; # p<0.05 vs HI+VEH; $ p<0.05 vs HI+CBD; & p<0.05 vs HI+HU-308 by Anova and t-test.*

HI led to an impairment of motor reflexes as observed one week after the insult in negative geotaxis, grip and grasp reflex (Fig. 45). CBD administration reduced HI-induced impairment in grasp test with no effect observable in the other tests (Fig. 45C). HU-308 showed no beneficial effect in any reflex test performed (Fig.45). Surprisingly, the co-administration of CBD and the CB_2 agonist led to an additive protective effect in the grip reflex test (Fig. 45B). To further assess the role of CB_2 receptor in CBD protection, the specific antagonist SR2 was administrated along with the previous treatment. The CB_2 antagonist administration prevented the protective effect showed by CBD (Fig. 46).

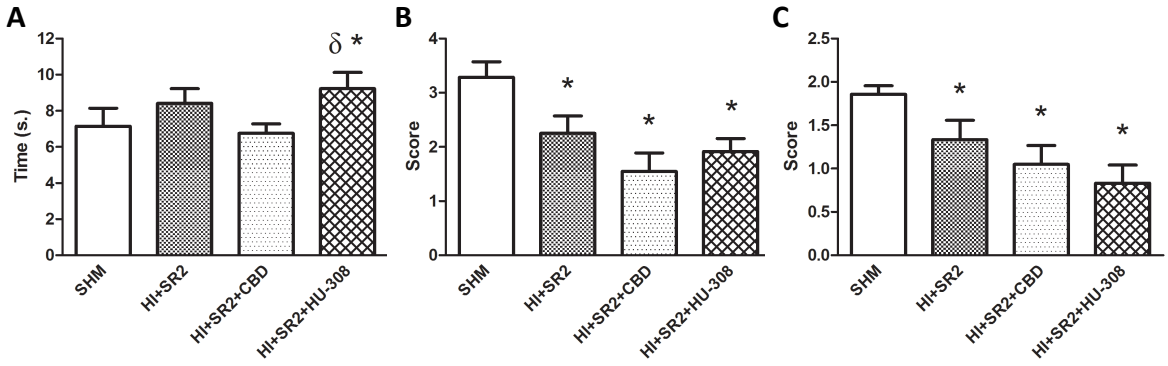


Figure 46. SR2 effect on motor disability-induced by HI. Analysis of motor impairment seven days after HI, after what animals were treated with CB_2 antagonist SR2, SR2 plus CBD or SR2 plus HU-308. **A)** Negative geotaxis; **B)** Grip reflex and **C)** Grasp reflex Bars represent mean±SEM. ** p<0.05 vs its SHM; δ p<0.05 vs HI+SR2+CBD by Anova and t-test.*

CBD reduced both the volume of brain damage and the hyperintense area, an effect not observable after HU-308 administration (Fig. 47A and C). In this case the administration of HU-308 together with CBD did not significantly reduce neither the volume of lesion nor the hyperintense area (Fig. 47A and C). CB_2 antagonism had no effect on brain damage or hyperintense area volume (p=0.123 and p=0.938 vs HI+VEH, respectively) (Fig. 47B and D). Interestingly, CB_2 antagonist co-administration did not modify the effects of CBD on the volume

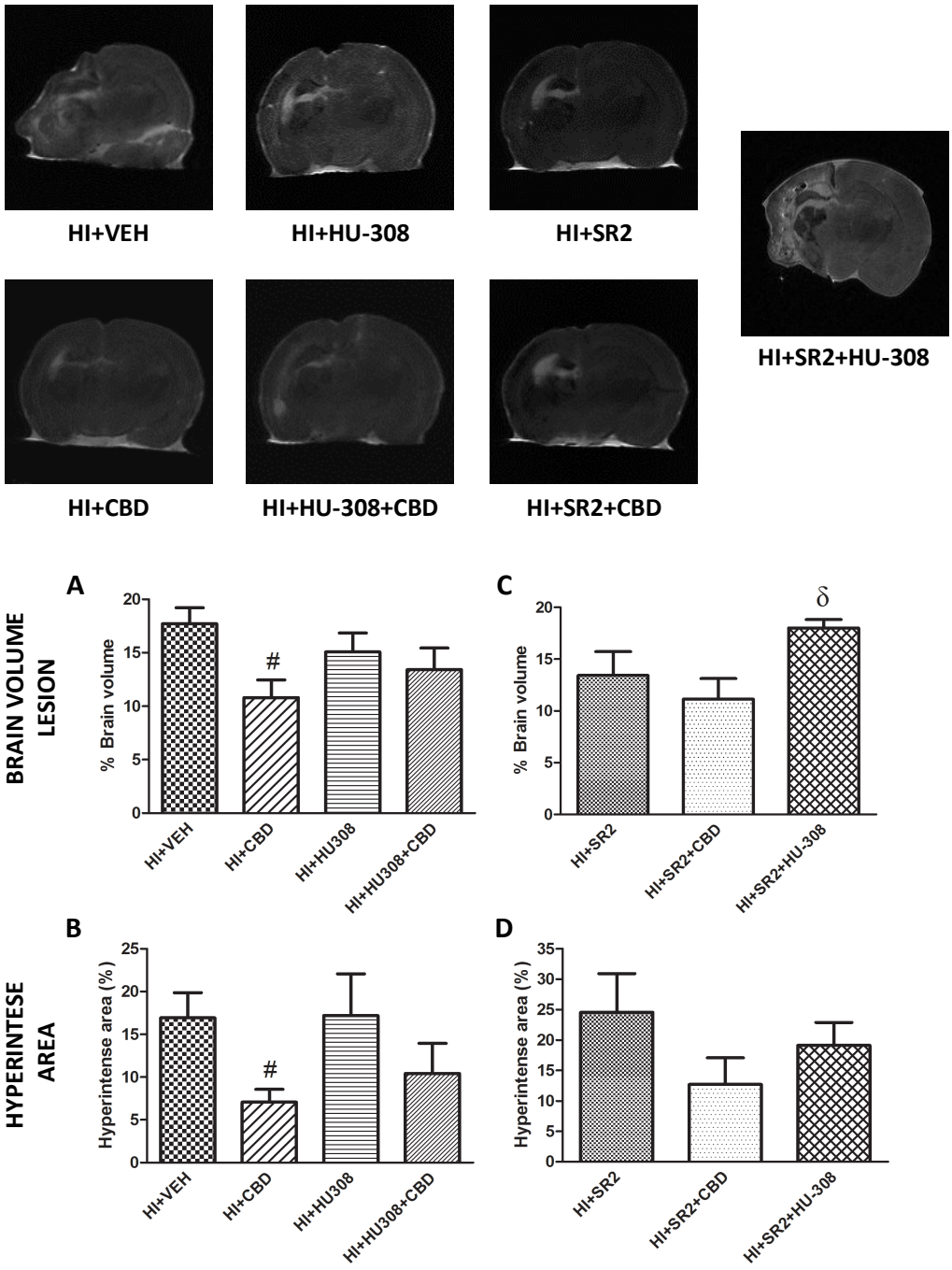


Figure 47. Volume brain damage evaluation. MRI Studies conducted in Wistar rats seven days after PAIS in animals treated either with CBD or HU-308 (**A** and **B**) or with SR2 (**C** and **D**). Both the total brain volume lesion and hyperintense are were analyzed. Bars represent mean±SEM. *# p<0.05 vs HI+VEH; δ p<0.05 vs HI+SR2+CBD by Anova and t-test.*

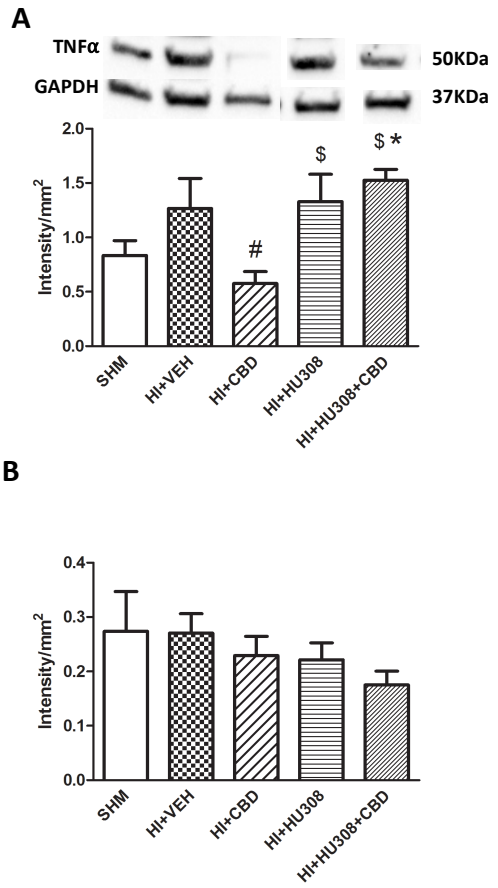


Figure 48. CBD and HU-308 effect on inflammatory and oxidative stress response in cortex after HI. Western-blot tests performed in the ipsilateral cortex of Wistar rats one week after PAIS. **A)** TNFα/GAPDH quantification and representative bands of TNFα trimer (50KDa) and GAPDH (37KDa); **B)** Oxyblot quantification normalized by total protein loaded. Bars represent mean±SEM. ** p<0.05 vs SHM; # p<0.05 vs HI+VEH; $ p<0.05 vs HI+CBD; by Anova and t-test.*

of lesion (p=0.023 vs HI+VEH) (Fig. 47C) but blunted the effects of CBD on the hyperintense area volume (p=0.474 vs HI+VEH) (Fig. 47D).

Finally, similar results were obtained when the protein levels of TNFα were studied by western-blot. CBD prevented the increase of this cytokine induced by HI; that protective effect was reduced by CB_2 antagonist co-administration (p=0.533 vs HI+VEH) (Fig. 48A). The administration of HU-308 did not reduced the TNFα increase induced by HI (Fig. 48A) an effect that was not modified by the co-administration of CBD and HU-308 (Fig. 48A). No oxidative stress differences were observed among the different groups (Fig. 48B and 49B).

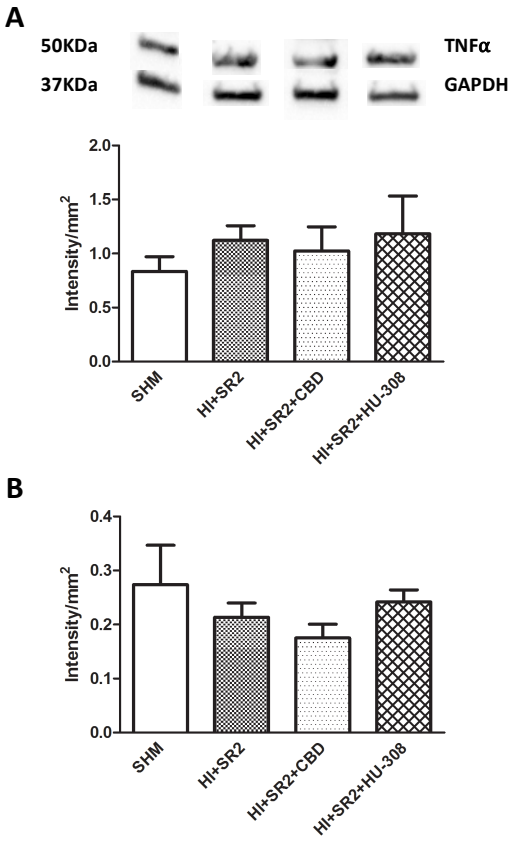


Figure 49. Analysis of inflammatory and oxidative stress in cortex after a HI event and the SR2 administration. Western-blot assay performed in the ipsilateral cortex of Wistar rats seven days after PAIS and posterior treatment with SR2, SR2+CBD and SR2+HU-308. **A)** TNFα/GAPDH quantification; **B)** Oxyblot quantification normalized by total protein loaded. Bars represent mean±SEM.

4.1 Summary

Only CBD administration was able to reduce motor impairment, brain volume damage, as observed by MRI, and brain TNFα concentration increase. The administration of HU-308 only showed some protective effect in motor test but not in MRI or biochemical studies. The co-administration of HU-308 with CBD potentiated the CBD protective effects in motor test but not as long as brain damage volume or brain TNFα levels was concerned. The administration of both CBD and SR2 reversed some CBD protective effects, which might indicate that this receptor is somehow involved in CBD neuroprotective actions.

Discussion

Neonatal hypoxic-ischemic brain injury is a severe pathology with deleterious consequences like cerebral palsy, which lasts for the rest of the life of the individual and the family (Adhikari and Rao, 2016; Martinez-Biarge et al., 2011; Nelson and Lynch, 2004). Unfortunately, the only treatment currently used, hypothermia, is only partially effective in cases of moderate to severe NHIE and with a very short therapeutic window; for PAIS current treatment is only symptomatic (Armstrong-Wells and Ferriero, 2014; Cnossen et al., 2009; Sagredo et al., 2018). Therefore, there is an actual need for new drugs that can improve neonatal hypoxic-ischemic brain injury outcome; in this regard, CBD has risen as a promising therapeutic drug (Fernández-Ruiz et al., 2015; Martínez-Orgado et al., 2007; Sagredo et al., 2018). Different works from our group have already proven that CBD is neuroprotective in *in vitro* and *in vivo* models of global HI (Castillo et al., 2010; Lafuente et al., 2016, 2011, Pazos et al., 2013, 2012), with remarkable beneficial effects in functional/ neurobehavioral studies (Lafuente et al., 2011; Pazos et al., 2012).

1. CBD NEUROPROTECTIVE EFFECTS IN A FOCAL MODEL OF HYPOXIC-ISCHEMIC BRAIN INJURY IN NEWBORN RATS

In this work we have demonstrated that the induction of HI or MCAO in newborns rat pups of PND7-10, led to brain injury with long-lasting sequelae as analyzed seven or thirty days after the insult that were mostly prevented by CBD administration ten minutes after the insult. Surprisingly CBD was neuroprotective at such a low dose as 1 mg/kg in both models, differently to what has been reported in adult chronic neurodegenerative disease models (Cheng et al., 2014; Sagredo et al., 2007). Hayakawa et al. have reported that two doses of at least 3 mg/Kg, one of them during occlusion, are needed to obtain CBD neuroprotective effects in an adult model of MCAO (Hayakawa et al., 2007b, 2007a). By contrast, in our neonatal MCAO model we obtained neuroprotective effects with CBD single dose administered only post-stroke. In that neonatal model better results were obtained with a dose of 5mg/Kg than with 1mg/Kg: higher doses did not significantly improve neurobehavioral tests or MRI analysis of brain damage results. In fact, the dose of 100mg/Kg increased the mortality rate. Conceivably, since CBD at high doses can induce hypotension (Jadoon et al., 2017) the administration at such a high dose in our model might have potentiated the blood pressure decrease due blood loss often observed during the surgical procedure.

Both global and focal ischemic-brain damage induced motor reflex impairment seven days after damage, which was prevented by post-insult CBD administration in MCAO but not in HI models,

where only a reduction in the grasp reflex was observed. That could be explained not only by the higher dose used in MCAO model but also for the severity and the different areas affected by the HI damage. While focal HI, i.e. MCAO, led to damage circumscribed to the MCA territory affecting cerebral cortex and caudate nucleus (Derugin et al., 2000, 1998), global HI induced a diffuse wide lesion involving cerebral cortex, subcortical and periventricular White Matter, deep Grey Matter structures like basal ganglia and thalamus, striatum and hippocampus (Vannucci and Vannucci, 2005). Therefore, the lower dose administrated in HI model might have not been effective enough to reduce the motor impairment observed early after the insult, whereas in the model of MCAO CBD could have been more effective taking into account that the dose was higher and the brain damage area smaller.

In agreement with the different brain areas affected, results from the neurobehavioral tests observed thirty days after damage were also different. Both models induced a long-lasting motor impairment showed by hemiparesis and motor coordination loss, as previously reported in HI models (Vannucci and Vannucci, 2005). This motor disability correlates with the disruption of M1 motor cortex connectivity (Gharbawie et al., 2008) which could have happened in both models. By contrast, HI led to a cognitive impairment which was not observed in the MCAO model. As explained before, HI causes great damage on hippocampus (Vannucci and Vannucci, 2005) which could affect the memory-related performance as explored using the NOR test (Antunes and Biala, 2012). However, since Middle Cerebral Artery does not irrigates hippocampus (Gharbawie et al., 2008, 2005), MCAO-induced insult are not inducing the same effects on memory. Mechanisms of CBD long-term neuroprotective effects will be discussed later in this section.

In our MCAO model early poor motor reflex performance demonstrated a good correspondence with the definitive adult motor impairment, as previously reported (Ten et al., 2003). In the HI model, however, CBD protective effects were more apparent one month than one week after the damage. That effect can be related to the well-known evolution in space and time of the more diffuse damage observed in HI models (Mehta et al., 2007; Uria-Avellanal and Robertson, 2014). In agreement, the relative brain volume loss area induced by HI as observed by MRI in rats decreases from one week to one month after the insult (Pazos et al., 2012). By contrast, the MCAO-induced relative brain volume loss area observed in our experiment was not only smaller than in HI but it also remained similar one week and one month after the insult.

Despite a similar degree of brain volume loss in MCAO-VEH and MCAO-CBD, CBD administration significantly decreased the hyperintense area as compared to MCAO-VEH, at both time-points.

This hyperintense area corresponds at least in part to vasogenic and cytotoxic oedema during the first days after the stroke (Allen et al., 2012; Qiao et al., 2007), but subsequently it correspondes to an area of gliotic scar and cell debris accumulation (Justicia et al., 2008; Velthoven et al., 2017). This hyperintense area correlates with the increased GFAP staining observed one week but mainly one month after the insult. Indeed, it has been described that astrogliosis induced shortly after a HI insult will eventually produce the so-called "glial scar" (Shrivastava et al., 2012; Wang et al., 2012) which corresponds to that observed by MRI one month after MCAO and that was decreased by CBD administration Noteworthy, although the purpose of this biological response is undoubtedly to delimitate brain damage (Bush et al., 1999; Wanner et al., 2013), the composition and dimension of this glial scar has been inversely correlated with the survival of perilesional neuron networking; being one of the main differences between adult and neonatal positive outcome after stroke (Teo et al., 2018). Thus, CBD reduction of hyperintense area and glial scar observed one month after MCAO might be a factor contributing to neuron loss prevention and better neurobehavioral outcome.

As previously reported HI brain injury induced a strong inflammation response characterized by a remarkable micro- and astrogliosis. In both models, a sharp increase of astrocytes and microglia cellularity was observed after the insult, as described before (Buser et al., 2012; Hellström Erkenstam et al., 2016; Huang et al., 2017; Liu and McCullough, 2013; McRae et al., 1995; Shen et al., 2012). Those glial cells also presented an activated morphology. This pro-inflammatory state of glia was confirmed by the significant increase of inflammatory cytokine TNFα in HI model as well as the high oxidative stress levels observed. The increase of both TNFα and oxidative stress were dramatically blunted by CBD administration.

In astrocytes the activated state is characterized by the increase of GFAP intensity staining and a morphological change with shorten and engrossed processes (Cengiz et al., 2014; Diaz et al., 2017). We observed that response mainly in the HI model. These activated astrocytes are known to promote inflammation by releasing cytokines and other products triggering death signals (Faustino et al., 2011; Hagberg et al., 2015; Jin et al., 2009; Kichev et al., 2014; Liu and McCullough, 2013). MCAO astrogliosis was rather characterized by a loss of astrocyte function, as reflected by the decrease of mI/Cr ratio (Harris et al., 2015). This is important because astrocytes play a protective role after HI reducing the volume of damage (L. Li et al., 2008), promoting neurites growth (Wang et al., 2012) and keeping glutamate uptake and thus decreasing excitotoxicity (Cengiz et al., 2014; L. Li et al., 2008). CBD administration reduced astrogliosis in both HI and MCAO models, not only reducing the number of astrocytes but also

reducing its pro-inflammatory state or preserving its function. To our knowledge, this glio-protective effect has not been reported in other treatments.

HI and MCAO insult led to an increase in microglia cell number. Those cells showed an ameboid phenotype with reduced ramification length, a phenotype corresponding to activated microglia (Kaur et al., 2013). Although CBD administration only partially reduced microglia proliferation in the MCAO model, that treatment was associated with a reduction of the presence of a non-amoeboid microglia phenotype, suggesting a reduction of microglial activation. CBD anti-inflammatory effect has been largely described (Khaksar and Bigdeli, 2017; Mukhopadhyay et al., 2011; Vilela et al., 2017), including neonatal hypoxia-ischemia and adult stroke models (Khaksar and Bigdeli, 2017; Lafuente et al., 2011; Pazos et al., 2012). Microglia plays a chief role containing brain damage after stroke (Faustino et al., 2011; Fernandez-López et al., 2016). Thus, indiscriminate microglial inhibition produces a more intense pro-inflammatory response, higher levels of brain volume damage and haemorrhages post-stroke (Faustino et al., 2011; Fernandez-López et al., 2016). Noteworthy, CBD promotes microglial phagocytosis via TRPV1 receptor activation (Hassan et al., 2014), thus contributing to damage reduction. Summarizing, CBD administration post-damage would promote a repairing microglia phenotype which would have a role in the neuroprotective effect of CBD and, consequently, in the motor improvement observed in the MCAO-CBD or HC group.

The fact that CBD was effective in modulating microglial cells in MCAO but no in HI model could be to the different role of macrophage infiltration in those conditions, a factor that deserves consideration since Iba-1 labels both microglia and macrophage (Matsumoto et al., 2008; Ohsawa et al., 2000). Whereas early BBB break after HI damage is a well-known phenomenon (Ek et al., 2015), recently it has been proved that BBB remains stable after a neonatal stroke (Fernández-López et al., 2012). Thus the macrophage infiltration component might be different in MCAO and HI (Fernández-López et al., 2012; Moretti et al., 2015; Vexler and Yenari, 2009).

Another very interesting feature was that microgliosis was still observed one month after damage in the MCAO model. Although it has been proposed that this prolonged immune response is mainly an adaptive mechanism (Hagberg et al., 2015; Shrivastava et al., 2012; Winerdal et al., 2012), such a long-term microgliosis could be particularly harmful, not only because of the deleterious effect of ROS and cytokine release, but also because “resting” microglia plays a physiological role of pruning neuronal contact and define neuron circuitry during development (Low and Ginhoux, 2018; Mallard et al., 2018; Schafer et al., 2012), an effect potentially lost when microglia changes into an activated state.

Another important factor of CBD neuroprotection was the decrease of excitotoxicity induced by MCAO or HI as observed short-term after the damage. HI/MCAO-induced excitotoxicity is increased by neuron delayed death, still observed one week after insult, but also by the enhanced glutamate release by reactive astrocytes and microglia (Cengiz et al., 2014; Hagberg et al., 2015; Socodato et al., 2018) as well as an impaired astrocyte re-uptake (Cengiz et al., 2014; Swanson et al., 1995). CBD excitotoxicity modulation after hypoxic-ischemic insults has been previously reported (Castillo et al., 2010; Pazos et al., 2013, 2012) and would be likely contributing to the brain damage reduction observed in MCAO-CBD or HC.

In agreement with CBD reduction of inflammation, oxidative stress and excitotoxicity, CBD administration reduced cell death, blunting neuronal loss in perilesional cerebral cortex and modulated post-insult increase of the levels of Lac/NAA, a marker of metabolic derangement and tissue damage (Li et al., 2010; Pazos et al., 2013, 2012; Penrice et al., 1997b). Similar neuroprotective results were previously obtained in HI model (Pazos et al., 2012), although CBD was not able to reduce the number of dead cells one week after such a diffuse damage.

2. HI-INDUCED LONG-LASTING HYPOMYELINATION IN WHITE AND GREY MATTER AND ITS PREVENTION BY CBD

Beside the neuronal loss, hypomyelination was also detected one month after the insult in both models, correlating with the early loss of myelin fibers and the accumulation of myelin debris reported in the first weeks after neonatal stroke (Velthoven et al., 2017; Villapol et al., 2011). This would be particularly important since several studies have pointed out that myelin loss might also been important in HI and PAIS motor and cognitive impairment development (Choi et al., 2018; de Vries and Jongmans, 2010; Groeschel et al., 2017; Martinez-Biarge et al., 2011; Stewart et al., 2017). By contrast, CBD administration post-insult reduced hypomyelination as well as prevented motor and cognitive impairment.

The relationship between long-lasting sequelae and hypomyelination can be explained by several factors. Firstly, by the hypomyelination itself. In both corpus callosum and parieto-temporal cortex we could observe an increase of the g-ratio due to a smaller myelin thickness; that thinner myelin sheath has lower conduction velocity (Clayton et al., 2017; Drobyshevsky et al., 2014). In fact, a promising theory has stablished that myelination is another kind of brain plasticity, promoting those mostly activated circuits (Mitew et al., 2016; Stedehouder et al., 2018). Secondly, by the loss of axons observed in both areas, probably due to the higher sensitivity of poorly myelinated axons to damage (Lee et al., 2012; Nave, 2010). Actually, human

newborns showing a similar myelin damage in cortex and watershed White Matter develops hemiplegia or cognitive impairments (de Vries and Jongmans, 2010; Martinez-Biarge et al., 2011), consequences comparable to those observed in our experiment. In our experiment, White Matter hypomyelination correlated with hemiparesis, in agreement with the correspondence of lower fractal anisotropy and volume of corpus callosum tracts with hemiplegia in humans after adult or neonatal stroke (Groeschel et al., 2017; Stewart et al., 2017). Besides, the long-lasting cognitive impairment observed in our rats was directly related to low myelin levels in parieto-temporal cortex, a region involved in working memory (Yoon et al., 2008) and which damage induces spatial memory impairment (Zhou et al., 2016).

HI-induced axonopathy in both areas deserves more deeply studies. In cortex, axon diameter was similar in all groups despite the fact that HV animals showed oligodendrocyte loss and axon density reduction likely because of the aforementioned higher sensibility of unmyelinated axon to damage (Lee et al., 2012; Nave, 2010). By contrast, in White Matter the specific loss of bigger diameter axons could be caused by its particular sensitiveness to excitotoxicity and therefore to HI-induced damage (Alix et al., 2012). In this scenario, glutamate reduction by CBD would help to protect axons from direct damage by HI. Consequently, CBD would help to maintain a proper myelination and oligodendrocyte maturation since axon-oligodendrocyte interaction is necessary for some axon myelination (Lundgaard et al., 2013; Mitew et al., 2016; Shen et al., 2012).

Although we did not obtain a statistically significant correlation between MBP signal in both areas and the improvement observed in HC group in the neurobehavioral tests, it is conceivable that CBD beneficial effects on motor and cognitive impairment might rely at least in part on a protective effect on myelination. Supporting that suggestion is the fact observed in MCAO experiments, in agreement with that previously reported for HI (Pazos et al., 2012), that CBD was far more effective on preventing post-insult long-term functional disabilities than on reducing the volume of brain damage or hyperintense area or neuronal loss. Other treatments like hypothermia, melatonin or erythropoietin also reduce the MBP loss (Fan et al., 2011; Kida et al., 2013; Lee et al., 2016; Reinboth et al., 2016; Villapol et al., 2011; Xiong et al., 2013), although only hypothermia given immediately after HI was able to prevent hypomyelination as observed in both White Matter (Reinboth et al., 2016; Xiong et al., 2013) and cortex (Kida et al., 2013). However, comparisons should be done cautiously since results from all those studies might be influenced by the different species, strains and gender.

Myelination studies performed in the HI model point out that HI affects differently Grey and White Matter. While HI led to the reduction of myelin sheath thickness and number of axons in both areas, the more dramatic loss of myelin and mature oligodendrocyte was observed in cerebral cortex. Different responses depending on the specific area can be explained by the different characteristics displayed by oligodendrocytes in White or Grey Matter (Lentferink et al., 2018; Viganò et al., 2013). Those cells respond differently to astrocytic cues or inflammatory cytokines depending on the area (Lentferink et al., 2018) and differentiation of OPC in White Matter is more intense and quicker than in Grey Matter (Lentferink et al., 2018; Viganò et al., 2013). Several studies point to the different maturational stage of oligodendrocyte in Cortex and White Matter at term (Buser et al., 2010; Segovia et al., 2008; Tomassy and Fossati, 2014) as the main reason for those regional differences. Whereas White Matter oligodendrocyte maturation from preOL to iOL triggers between 18 and 27 postconceptional week in human (equivalent to PND2-PND3 in rats) (Back et al., 2002a, 2002b), cortex preOL maturation happens around birth at term (equivalent to PND7-PND10 in rats) (Segovia et al., 2008; Tomassy and Fossati, 2014). Therefore, due to the special sensitivity of preOL but not iOL to the "deadly triad" characteristically observed after HI (Alix, 2006; Back et al., 2007; Back and Rosenberg, 2014; Fragoso et al., 2004; Volpe, 2011), the deleterious effects of HI occurring at term (or equivalent period in rats) on OL maturation should take place mainly in cortex but not in White Matter. In agreement, studies on very preterm babies, whose White Matter is populated mainly by preOL (Back et al., 2002a; Buser et al., 2010), have revealed that severe preterm White Matter pathology such as periventricular leukomalacia, originated by brain insults occurring before the 27th postconceptional week, is characterized by myelin loss in the White Matter with reduced numbers of mature oligodendrocyte (Segovia et al., 2008). Hence, effects on White Matter of brain insults occurring over the period of preOL maturation in that area in humans were similar to those observed in our experiments in cortex after brain insults occurring over the time point where maturating preOL are the main oligodendrocyte population in this area (Back et al., 2002a; Dean et al., 2011).

PreOL particular sensitivity to HI damage is caused at least in part by the activation of AMPA receptors localized in soma (Follett, 2004; Salter and Fern, 2005). The over-activation of AMPA receptors by the high levels of glutamate observed after HI together with the increased permeability of the receptor in neonatal brain (D M Talos et al., 2006; Delia M Talos et al., 2006), induces a remarkable increase of intracellular calcium leading to cell death (Follett, 2004; Shen et al., 2012). PreOL are also quite vulnerable to oxidative stress because of the low concentrations of GSH associated with a high intracellular concentration of iron (Thorburne and

Juurlink, 1996), required for myelin synthesis (Connor and Menzies, 1996), whose homeostasis is impaired after HI (Buonocore et al., 2001). Inflammation is also very harmful to preOL. PreOLs have TNFα receptors in the soma (Deng et al., 2008; Su et al., 2011) whose activation induces preOL death or inhibits its maturation (Su et al., 2011) by a mechanism mediated by astrocytes (Kim et al., 2011). Besides, they are particularly sensitive to HI-induced astro- and micro-gliosis that inhibit oligodendrocyte maturation by modifying neurotrophic factor secretion (Wang et al., 2011). Moreover, iron accumulation in activated microglial cells, like the ones observed in our model, induces a decrease of glutathione and potentiates lipid peroxidation in oligodendrocytes (Rathnasamy et al., 2011). Reactive astrocytes secretes hyaluronic acid, a potent inhibitor of both myelination and OPC maturation, to create the gliosis scar described in our experiments (Buser et al., 2012).

Despite the fact that White Matter was populated mainly by iOL at the moment of the HI insult in our experiments, and that iOL are more resistant to HI effects than preOL, myelination at White Matter was also affected. This was probably happening because myelination is still in progress in White Matter at the time when birth takes place at term with iOL extending their processes to axons and forming the myelin sheath (Back et al., 2002a). This process could be affected by excitotoxicity and neurotrophic factors, among others (Jantzie et al., 2015; Miron et al., 2013a). Noteworthy, iOL express NMDA receptors in their processes to get axonal inputs (Salter & Fern, 2005; Follett, 2004). Those NMDA receptors are also highly sensitive to glutamate (Ewald and Cline, 2009; Kirson et al., 1999). Because of that, the high levels of glutamate that last even a week after the insult will be particularly harmful for those oligodendrocytes processes. Intracellular calcium concentration would rise exponentially in iOL processes, inducing local excitotoxicity which can damage the processes without affecting the viability of the cell (Follett, 2004; Salter and Fern, 2005; Volpe, 2009). That would explain the myelin defects observed in in the second goal of our experiments in spite of the preservation of mature oligodendrocyte cell density.

Summarizing, because of the increase oxidative stress, glutamate and inflammation observed seven days after the insult, preOL population was reduced in Grey Matter after HI whereas White Matter iOL oligodendrocyte survived after the insult, although with reduced myelinating activity. CBD administration was able to prevent both myelination impairment in White Matter and hypomyelination and mature oligodendrocyte loss in cortex. This work demonstrates by the first time that CBD has beneficial effect on oligodendrocytes and myelination after acute neonatal brain injury, an effect in which CBD reduction of the “deadly triad” as well as the

modulation of astro- and microglial activation are likely playing key roles. CBD has proven to protect both astrocyte and neuron after neonatal damage in this work and others (Lafuente et al., 2016, 2011; Pazos et al., 2013) and shows oligoprotective properties in animal models of Multiple Sclerosis (Mecha et al., 2012; Rahimi et al., 2015).

3. ASSESSING IF CBD HYPOMYELINATION PREVENTION IS RELATED WITH BRAIN CELL PROLIFERATION ACTIVATION AND GLIAL RESPONSE MODULATION

Beside its effects on cell death signals, HI also triggers a proliferative response (Kido and Matsumoto, 2016; Ong et al., 2005; Plane et al., 2004; Zhu et al., 2009). Actually, enhanced OPC proliferation has been characterized in models of preterm brain damage, although the maturation process of those cells is interrupted, remaining in an "arrested preOL stage" and eventually leading to hypomyelination (Buser et al., 2012, 2010; Segovia et al., 2008).

The proliferative response was strong in the SVZ, one of the main proliferative niches of brain and responsible for generating OPC that lately migrate and differentiate in the different White Matter areas (Mao et al., 2013). Puzzling, we observed that HI induced an increase in the number of proliferating cell 24 hours after the insult in the SVZ, as has been reported (Ong et al., 2005; Plane et al., 2004), but in our experiments that number was restored and even decreased in HV group one week later. It has been described that HI-induced proliferation is localized in specific areas of the SVZ, so that the effect can be overlooked when the entire SVZ area is analyzed (Felling et al., 2006). However, we cannot rule out the possibility that our different results were due to different species and HI protocol used. The oligo-proliferative response was also observed in our model in both corpus callosum and SVZ: the population of OPC and preOL, reduced 24 h after the HI insult, was restored seven days later. The increase in preOL population observed in HV as compared to SHM group agreed with the well-known compensatory increase of preOL described in models of preterm White Matter injury (Back et al., 2002a; Buser et al., 2012; Segovia et al., 2008). By contrast, the increment of OPC in corpus callosum is not observed after HI damage in models of term brain injury (Dizon et al., 2010). Altogether those features suggest that the kind of proliferative response of oligodendrocyte is dependent on the developmental stage.

Surprisingly, no CBD effects on oligodendrocyte progenitor or proliferation itself were observed that could explain CBD prevention of hypomyelination, with the exception made for the increase of Ki67-positive cell density observed one week after the insult in HC group. Similar results has been observed in HI newborn rats treated with hypothermia after co-localizing KI67 with an oligodendrocyte precursor marker (Xiong et al., 2013). Since no differences were observed in the number of Olig2 or Sox10 among the different groups, it is unlikely that HC-induced proliferative enhancement in White Matter corresponded to increased proliferation of OPC. Rather, other cellular types should contribute to this CBD-induced increased proliferation. Some candidates are astrocytes or immune cells whose proliferative response as has been characterized in other brain regions (Domowicz et al., 2018; Kido and Matsumoto, 2016; Zhu et al., 2009). Nevertheless, a more detailed study of oligodendrocyte maturation stages and its origin, as well as other White Matter lesion area characterization should be performed to further characterize this response.

Also in agreement with what has been previously reported (Kido and Matsumoto, 2016; Zhu et al., 2009), the quantification of Ki67-positive population in cerebral cortex showed increased proliferation seven days after HI. However, when BrdU studies were performed in perilesional cortex no difference was observed among groups seven days after the insult. This divergence between Ki67 and BrdU studies might be explained because these techniques label different cell populations: Ki67 is a nuclear protein which is mainly expressed during cell division phases, so that its antibody labels actual proliferating cells (Boddaert et al., 2018; Gerdes et al., 1991). By contrast, BrdU is an analogue nucleotide which is used to track cells which were created during BrdU injection period (Fernández-López et al., 2010; Magavi and MacKlis, 2008). According to this, the aforementioned results would suggest that although there was an increased proliferative activity exactly seven days after the insult, no increased proliferation took place during the fourth, fifth and sixth days post-insult. The lack of increased proliferation during the first days after the HI episode might be due to increased cell death that would limit the survival of these new cells. In agreement with this hypothesis, a remarkable rise of TUNEL-positive cells was observed in HV and HC groups. Similar results were found an *in vitro* model of HI, where the number of $BrdU^+$ cells were significantly lower in OGD than in the control group (Ziemka-Nalecz et al., 2018). The double-edged effect of HI on proliferation was also observed in BDNF studies, in which an increase of both pro and mature isoforms of BDNF was observed one week after HI independently of the treatment. The positive effects of BDNF, like neuron proliferation and protection, are associated to the mature form (Jiao et al., 2016; Li et al., 2017); while the precursor one, proBDNF, is known to limit neuron proliferation and migration (Li et al., 2017).

Similar results to that obtained in White Matter was observed in cortex, in which a normalization of Olig2$^+$ and SOX10$^+$ population was observed seven days after HI. At this time-point no changes in IGF-1 or GDNF brain concentration was found. Those molecules have well-known oligodendrocyte proliferating and maturating effects (Broughton et al., 2007; Li et al., 2015; Shi et al., 2014). The quantification of Ki67$^+$/Olig2$^+$ cells, corresponding to proliferating OPC, confirmed that oligodendrocyte lineage was not being promoted seven days after damage, despite the observed increase of proliferating cells. These results might suggest that a compensatory oligodendrocyte proliferative response took place early after the insult to restore oligodendrocyte cell density, resulting in no more oligodendrocyte proliferation happening one week after damage. Supporting this hypothesis, a reduction of BrdU$^+$/SOX10$^+$ cells was also observed in HV animals despite of SOX10$^+$ population normalization implying that HI induced not only an early proliferative but also a quick differentiation of OPC. That is particularly interesting because the "arrested preOL state" has been correlated to an early exit of the cycle in OPC that, somehow, affects the capacity of survival and maturation of preOL into a myelinating oligodendrocyte (Back et al., 2002a) (Fig. 50).

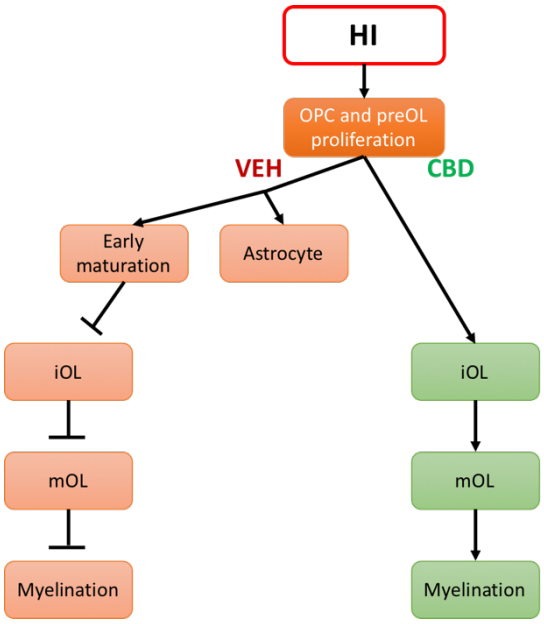


Figure 50. Oligodendrocyte proliferative response induced by HI hypothesis and possible effect of CBD in cortex. Oligodendrocyte progenitors proliferation is induced after HI but its maturation is impaired, eventually leading to hypomyelination. The administration of CBD post-HI is able to prevent this oligodendrocyte arrested state and consequent hypomyelination. OPC, oligodendrocyte progenitor cell; preOL, pre-oligodendrocyte; iOL, immature oligodendrocyte; mOL, mature oligodendrocyte.

The aforementioned results also suggest that HI-induced proliferation observed in HV group seven days after the insult might involve other cellular lineages. In agreement, HV group showed higher Olig2$^+$/GFAP$^+$ cell density that correspondence with astrogliosis. Thus, it seems that astrocyte lineage proliferation was promoted over oligodendrocyte proliferation seven days after the HI event, an effect similar to that reported after adult spinal cord damage (Wang et al., 2011) (Fig. 50).

As opposite to the lack of effect of CBD on oligodendrocyte lineage cell proliferation in White Matter, in cortex CBD administration induced a rise of BrdU$^+$/Olig2$^+$ population that leads to the increase of Olig2$^+$ cell

density seven days after HI. These newly formed OPC cells seems to be driven into an oligodendrocyte lineage as suggested by the reduction of $Olig2^+/GFAP^+$ and increase $BrdU^+/Sox^+$ ratios to levels similar to SHM. Therefore, CBD seems to promote and sustain the oligodendrocyte proliferation response, limiting the pro-astroglial fate of OPCs induced by HI. This can result eventually in the protection of a physiological maturation of oligodendrocytes, thus avoiding the premature differentiation that might contribute to the HI-induced oligodendrocyte "arrested state" and subsequent hypomyelination (Fig. 50).

We observed that HI led to a reduction of the density of $BrdU^+/NeuroD^+$ cell density. Since global density of $BrdU^+$ cells were similar in all groups, this means that HI increased the proportion of glial cells among the $BrdU^+$ cell population. In other words, it means that HI induced a pro-glial response, affecting normal cortical maturation. Such effect of HI was prevented by CBD administration, suggesting that the beneficial effect of CBD was also affecting the proliferation of non-glial cells as neurons.

Summarizing those results, the HI insult increases excitotoxicity, oxidative stress and inflammation leading to astro- and micro-gliosis that was associated with increased preOL death. Likely, due to this "deadly triad" prolonged feedback the reparative response after HI was also affected, with astroglial lineage being preferentially promoted. Similarly to what observed in MCAO model, CBD administration post-HI was able to reduce the three components of the deadly triad as well as modulate both astroglial and microglial response, resulting in a more anti-inflammatory or repairing glia phenotype, which is involved in OPC protection and oligodendrocyte differentiation (Miron et al., 2013b). Interestingly, the switch of glial sate towards an anti-inflammatory or rested state is associated with re- and myelination processes (Miron et al., 2013b; Moore et al., 2011).

Then, on the one hand, CBD would be promoting oligodendrocyte survival and maturation in cerebral cortex and protecting myelination in White Matter by reducing these detrimental HI events. On the other hand, CBD would be preserving the success of the repair response induced by HI promoting the proper maturation and myelination of the new oligodendrocytes. This would explain the protective effect of CBD on hypomyelination and axonopathy observed in White and Grey Matter and, therefore, the reduction of long-lasting sequelae induced by HI (Fig. 51).

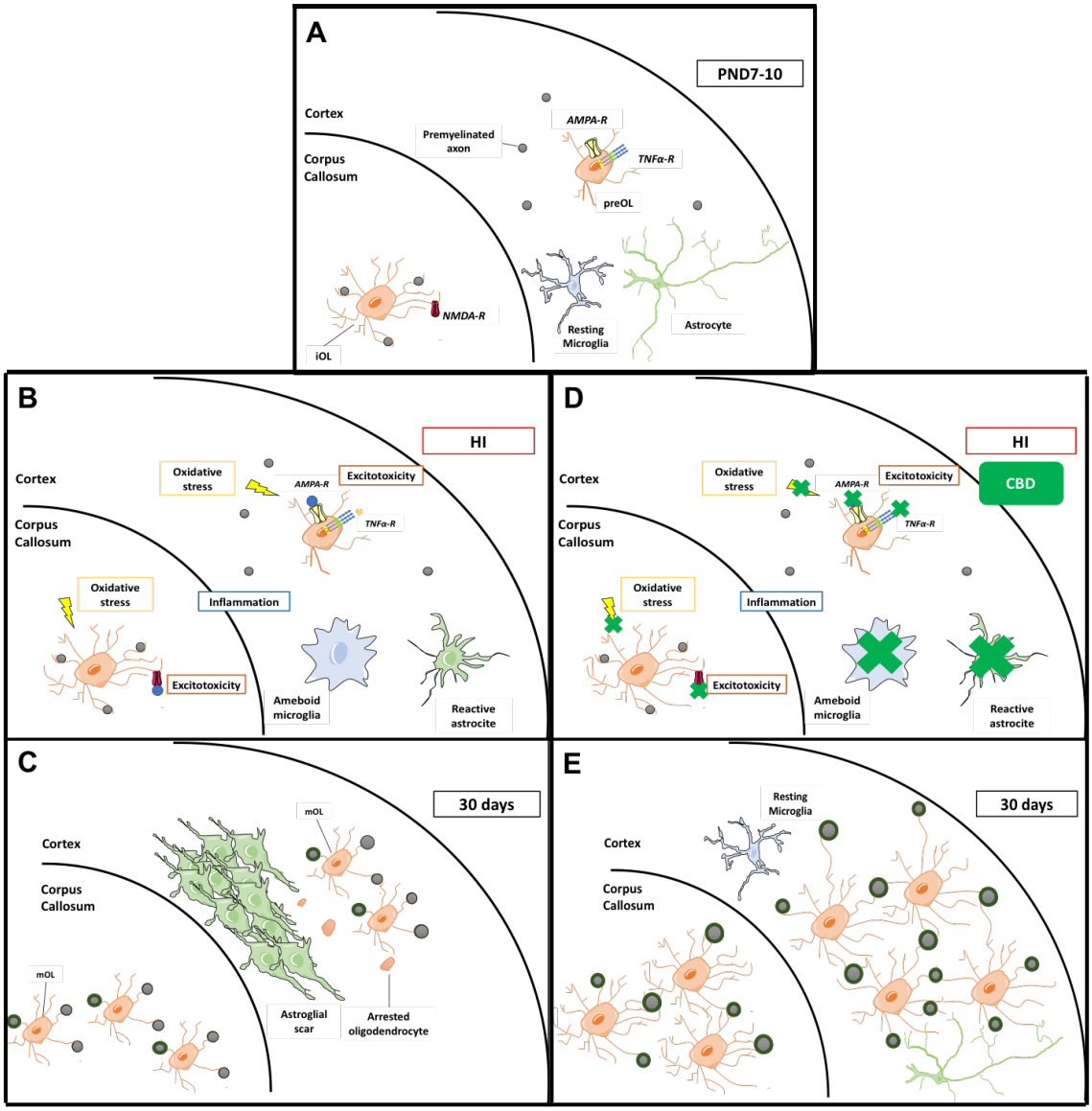


Figure 51. CBD prevents hypomyelination induced by HI. At PND7-10 cortex and White Matter are populated by oligodendroctyes at different maturational stages **(A)**. During HI the "death triad", a combination of excitotoxicity, inflammation and oxidative response, will damage oligodendrocytes of both areas **(B)**. In corpus callosum, the iOL processes failed to reach and myelinated axon while HI induceds cortical preOLs death. Eventually, this will lead to a decrease hypomyelination and axon loss in both areas **(C)**. By contrast, CBD administration is able to reduce these three components of the death triad **(D)**, eventually allowing a correct oligodendrocyte maturation and myelination and therefore, also preventing HI-induced axonopathy **(E)**.

4. INVOLVEMENT OF CB_2 RECEPTOR ACTIVATION IN CBD PROTECTIVE EFFECTS IN A NEWBORN RAT MODEL OF HYPOXIC-ISCHEMIC BRAIN DAMAGE

CB_2 agonists have proved neuroprotective effects in different models of neurodegenerative diseases, by similar glia-modulating and anti-inflammatory effects to those described for CBD in this work (Espejo-Porras et al., 2018; Fernández-Ruiz et al., 2007; López et al., 2018; Sagredo et

al., 2009; Tolón et al., 2009). These facts, together with previous reports stating that some of the protective effects of CBD are mediated by CB_2 receptor activation (Pazos et al., 2013; Vilela et al., 2017), prompted us to analyze the role of CB_2 receptor in CBD neuroprotection after a global hypoxic-ischemic insult in newborn rats.

After testing the neuroprotective effects of HU-308, we observed that CBD showed better neuroprotective effects than HU-308, which might be explained by the greater number of targets driven by CBD. CB_2 expression is thought to be limited to immune cells (Galiègue et al., 1995; Núñez et al., 2004; Turcotte et al., 2016), such as microglia (Núñez et al., 2004) and monocytes (Turcotte et al., 2016), which are the first infiltrating cells to become the predominant cell type present in brain in the first 24 hours post-insult (Hagberg et al., 2015; Winerdal et al., 2012). Therefore, HU-308 effects shall be restricted to these immune cells activation. The expression of CB_2 receptor in astroglial cells is more controversial as long as the physiological state is concerned (Dowie et al., 2014; Fernández-Trapero et al., 2017b), although CB_2 receptor expression has been detected in activated astrocytes in neurodegenerative conditions (Benito et al., 2007; Espejo-Porras et al., 2018; Sagredo et al., 2009). In any case, in our experiments HU-308 was unable to reduce neither the hyperintense area, which is at least in part due to the astroglial response one week after damage (Justicia et al., 2008) nor HI-induced TNFα production increase. Overall, these results suggest that CB_2 agonism would have a low effect on astroglia in a neonatal model of HI.

CB_2 receptor activation may be also involved in the anti-inflammatory effects of CBD. In this work, the administration of the CB_2 antagonist SR2 prior CBD injection reversed some of the CBD neuroprotective effects, in particular those related with motor reflex performance, brain volume of damage and hyperintense area. Since those results were associated to the reversal of CBD-induced TNFα release reduction by the pre-administration of SR2, it can be suggested that CB_2 receptor activation was somehow involved in the anti-inflammatory effect of CBD in the neonatal model of HI. Previous works of our group reported that CB_2 receptor activation was involved in CBD neuroprotection in a piglet model of acute HI brain damage (Pazos et al., 2013).

There are several explanations that might explain CBD and CB_2 interaction. A direct binding to the canonical site of CB_2 receptor can be ruled out since the concentration peak of CBD in brain after subcutaneous injection did not achieve the micromolar range needed for this interaction (McPartland et al., 2014; Pazos et al., 2012). However, binding studies are usually performed using human CB_2 receptor but binding characteristics of rodent and human receptor might be different (Shire et al., 1996), because of the poor inter-species CB_2 receptor homology (Brown

et al., 2002; Shire et al., 1996). This direct action on CB_2 receptor is very unlikely, however, because if CBD would act as a direct agonist of CB_2 receptor a similar neuroprotective effect would have been expected after HU-308 administration, particularly having into account the key role of the conversion of microglia cells and astrocytes into anti-inflammatory or non-activated states in the neuroprotective effects of CBD, as it has been extensively demonstrated in the present work.

Another possible mechanism could be the recently published activity of CBD as a negative allosteric modulator of the cannabinoid receptor (Laprairie et al., 2015; Martínez-Pinilla et al., 2017). That would be particularly interesting since the levels of AEA and 2-AG, which activate CB_2 receptor, are remarkably increased after a HI insult (Pazos et al., 2013), and 2-AG overexpression after stroke has been associated to a poorer outcome and microgliosis (Jalin et al., 2015; Shearer et al., 2018). Another hypothesis is that CB_2 and 5-HT_{1A} receptors could form an heteromer, like several other G protein-coupled receptors. The physical union of both receptors, which constitute the basis of an heteromer, has been proved in cells transfected with both receptors (Pazos et al., 2013). If such heteromer existed and was functional in newborn brain, the involvement of CB_2 on CBD actions might actually be the expression of a cross-agonism between CB_2 and 5-HT_{1A} receptors in the heteromer after CBD activated the 5-HT_{1A} (Russo et al., 2005). Nevertheless, further experiments are warranted to assess the role of CB_2 receptors in CBD neuroprotection. Those experiments should include the glial characterization, since glia modulation seems to play a pivotal role in CBD effect and CB_2 is highly expressed in those cells (Fernández-Trapero et al., 2017a; Núñez et al., 2004), as well as the study of other important immune cells involved like monocytes/macrophages, which infiltrate the brain after the insult and participate in damage evolution (Hagberg et al., 2015; Winerdal et al., 2012). Finally, another very interesting option will be to use a knock-out mouse model for CB_2 receptor.

CBD is also activating other receptors. Firstly, CBD shows a great affinity for 5-HT_{1A} receptors (Russo et al., 2005), whose activation by specific agonists after HI insults has been associated to a reduced brain volume of damage (Johansen et al., 2014) by reducing reactive astrocytes and limiting neuronal damage after the insult (Ramos et al., 2004) as well as by inducing hypothermia (Johansen et al., 2014). 5-HT_{1A} activation is involved in CBD positive hemodynamic effects (Pazos et al., 2013) and CBD protection of blood brain barrier stability (Hind et al., 2016). The latter results in a reduction of HI-induced brain oedema, an effect which could explain why CBD but not HU-308 reduced the hyperintense area as observed in MRI one week after damage. CBD anxiolytic effect as consequence of 5-HT_{1A} activation could represent an additional beneficial

effect for post-HI insult recover of the newborn (Fogaça et al., 2014). Another important receptor probably involved in CBD neuroprotective effects is the adenosine receptor A_2A (Carrier et al., 2006), which is expressed in microglia (Carrier et al., 2006), in neurons (Kerkhofs et al., 2018) and in astrocytes (Cervetto et al., 2018) and participates in the regulation of glutamatergic synapses (Cervetto et al., 2018; Kerkhofs et al., 2018). In an *in vitro* model of HI brain damage CBD A_2A receptor activation is involved in CBD-induced reduction of excitotoxicity and inflammation (Castillo et al., 2010). Also, CBD is a potent agonist of PPARγ (Hegde et al., 2015; O'Sullivan et al., 2009) which could explain an even stronger anti-inflammatory effect than CB_2 agonists. PPARγ is expressed in astrocytes (Chistyakov et al., 2015), microglia (Wen et al., 2018) and macrophages (Heming et al., 2018), neurons (Warden et al., 2016) and neural stem/progenitor cells (Yuan et al., 2017). PPARγ activation decreases inflammation (Villapol, 2018; Wen et al., 2018), promotes phagocytosis (Heming et al., 2018), stimulates that progenitor cells differentiate into oligodendrocytes or neurons instead astrocytes (Villapol, 2018; Yuan et al., 2017) and promotes the M2 phenotype in microglia (Wen et al., 2018), effects that could explain some of the results obtained in this study. Besides, PPARγ activation plays a key role in CBD effects on maintaining BBB stability after hypoxic-ischemic insult (Hind et al., 2016).

In this set of experiments no differences were observed regarding oxidative stress, which was probably due to the intrinsic variability of the experiment having into account that oxidative stress was reported in other experiments in this work as well as in previously reported similar HI neonatal models (Blanco et al., 2017; Pazos et al., 2013).

In conclusion, in a neonatal rat model of HI CBD administration was more effective than a direct CB_2 receptor agonist, with no additive effects after combining both substances. CBD neuroprotective effects were at least in part mediated by CB_2 receptor activation, which seems to be somehow involved in the CBD anti-inflammatory effects. The participation of CB_2 receptors in CBD effects has been already suggested in previous reports on neonatal HI brain damage, although the exact mechanisms involved has not been elucidated. Some hypothesis to explain why CB_2 receptors are involved in the effects of a substance like CBD, which is not considered a direct agonist of those receptors, include the existence of CB_2-$5HT_{1A}$ receptor heteromers in neonatal brain tissue after HI or the activity of CBD as an allosteric modulation of the cannabinoid receptor.

Conclusions

1. Neonatal hypoxic-ischemic (HI) insult led to brain injury resulting in long-lasting motor and cognitive sequelae, dependent on the areas affected by the insult. As observed one week after the insult HI events led to brain volume loss that correlated with acute cell death in a manner related with excitotoxicity, increased oxidative stress and inflammation. Therefore, HI led to a remarkable decrease on neuronal population and myelin content that were not recovered one month after the insult.
2. Global HI brain insult led to hypomyelination in White and Grey Matter, associated to axonal loss, which correlated with motor and cognitive disabilities. Myelination impairment was region-specific, probably because of the different oligodendrocyte maturational stage in the different regions.
3. Neonatal HI brain insult triggered a strong inflammatory response characterized by micro- and astrogliosis lasting for weeks after the insult. In both cases cell responses were characterized by the increase in the number of cells and the transformation of cells into an activated state. Activation of astrocytes led to a functional impairment and eventually led to the development of a glial scar, corresponding to an increased hyperintense area as observed by MRI one month later.
4. Post-insult CBD administration blunted the short- and long-term motor and cognitive impairment induced by either the global or the focal hypoxic-ischemic brain insult. CBD neuroprotective effects were linked to the preservation of neuronal population by reducing HI-induced cell death in a manner related with the reduction of excitotoxicity, oxidative stress and inflammation.
5. Post-HI administration of CBD also modulated the astroglial and microglial response, decreasing the gliosis and promoting a non pro-inflammatory state in both cell types as observed in the short- and long-term after the insult. Furthermore, CBD prevented the astroglial function impairment and the preferential development of glial precursors into astroglial instead of oligodendroglial lineage cells induced by the HI insult.
6. Post-insult CBD administration blunted the HI-induced hypomyelination and axonopathy in a region-specific manner. In all regions CBD treatment prevented HI-induced axonal loss and preserved the normal myelination of axons. In Grey Matter those effects were associated with the preservation of a proper maturation of immature glial cell whereas in White Matter they were associated with the preservation of the proper axon myelination by oligodendrocytes.
7. CB_2 receptor activation was involved in CBD neuroprotection after neonatal hypoxic-ischemic brain damage as suggested by the reversal of some neuroprotective effects of

CBD by CB_2 receptor antagonist. However, the comparison with the effects of pure CB_2 receptor agonists indicates that it is unlikely that CBD neuroprotection was due to a direct CB_2 activation and that the activation of such receptors cannot be the only way to explain CBD neuroprotective effects.

Conclusiones

1. El daño hipóxico-isquémico neonatal indujo un daño cerebral asociado a secuelas motoras y cognitivas a largo plazo, que van a depender de la zona afectada. Como se observó, el daño HI condujo a una pérdida de volumen cerebral asociada a una muerte celular aguda relacionada con la excitotoxicidad, el incremento del estrés oxidativo y la inflamación. En consecuencia, la HI produjo una disminución de la población neuronal y del contenido de mielina que aún pudo ser observado un mes después de la lesión.
2. El daño HI global indujo una hipomielinización en la Sustancia Blanca y Gris, asociada a la pérdida axonal y que se correlaciona directamente con la discapacidad motora y cognitiva. La alteración de la mielinización se produjo de manera distinta en cada región, probablemente por el diferente estado madurativo de los oligodendrocitos en cada área.
3. El daño HI neonatal promovió una fuerte respuesta inflamatoria caracterizada por una astro- y microgliosis que se prolongó durante semanas tras la lesión. En ambas poblaciones esta respuesta estuvo caracterizada por un aumento del número de células y por un fomento del estado activado. La activación astrocitaria condujo a una pérdida de función y, finalmente, al desarrollo de la cicatriz glial, que se correspondió con el incremento del volumen del área hiperintensa observado por resonancia magnética nuclear un mes después de la lesión.
4. La administración del CBD tras el daño previno el desarrollo de secuelas motoras y cognitivas a medio y largo plazo. Los efectos protectores del CBD se relacionaron con la preservación de la población neuronal mediante la reducción de la muerte celular causada por la HI, al reducir la excitotoxicidad, el estrés oxidativo y la inflamación.
5. La administración post-HI del CBD también moduló la respuesta astroglial y microglial, disminuyendo la gliosis y promoviendo el estado no pro-inflamatorio en ambos tipos celulares como pudo observarse a corto y largo plazo. Es más, el CBD previno la pérdida de la función astrocitaria y el fomento preferencial del linaje astrocitario sobre el oligodendrocítico, como consecuencia de la HI.
6. La administración de CBD tras el daño previene la hipomielinización y axonopatía de manera específica en cada región. En ambas áreas el tratamiento con CBD previno la pérdida axonal y permitió la correcta mielinización axonal. En Sustancia Gris estos efectos estuvieron asociadas a la preservación de la maduración de la glia inmadura mientras que en Sustancia Blanca se relaciono principalmente con el proceso de mielinización.
7. Se puede concluir que la activación del receptor CB_2 participó en la neuroprotección ofrecida por el CBD tras el daño HI, como sugiere la prevención de alguno de los efectos

protectores del CBD por la administración del antagonista del receptor. Sin embargo, la comparación con el agonista puro de CB_2 indica que es poco probable que la neuroprotección del CBD se deba a una activación directa del receptor y que debe haber más dianas o mecanismos implicados en la neuroprotección ofrecida por el CBD.

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Annex

INDEX OF FIGURES

Introduction

Methods

Results

CBD neuroprotective effects in a focal model of perinatal arterial ischemic stroke in newborn rats

Analysis of HI-induced long-lasting hypomyelination in White and Grey Matter and its prevention by CBD

Assessing if CBD hypomyelination prevention is related with brain cell proliferation activation and glial response modulation

Involvement of CB_2 receptor activation in CBD protective effects in a newborn rat model of hypoxic-ischemic brain damage

Discussion

INDEX OF TABLES

Introduction

Methods

Index

INDEX